Computational Modeling of the role of Adenomatous Polyposis Coli (APC) gene in the initiation of Colorectal Cancer

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Computational Modeling of the role of Adenomatous Polyposis Coli (APC) gene in the initiation of Colorectal Cancer"** is the original work done by me under the supervision of Dr. Elizabeth Jacob and the co-supervision of Dr. Savithri S., Computational Modeling and Simulation Group, Environmental Technology Division of CSIR – National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I also declare that this work did not form part of any dissertation submitted for the award of any degree, diploma, associateship, or any other title or recognition from any University/Institution.

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....but he has answered me, 'My grace is enough for you: for power is at full stretch in weakness.' It is, then, about my weaknesses that I am happiest of all to boast, so that the power of Christ may rest upon me. (2 Corinthians 12:9)

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"Not by might nor by power but by his grace"

PREFACE

Cancer remains one of the most complex diseases affecting humans. Despite identification of genetic mutations and aberrant signaling pathways, the process of carcinogenesis is incompletely understood. Adenomatous Polyposis Coli (APC) is a tumor suppressor gene which prevents the uncontrolled growth of cells that may result in cancer. Mutations in APC is found in 80% of colon cancers and is highly implicated as the initiating event of cancer. APC protein plays a critical role in the regulation of cellular processes like cell proliferation, migration, division, and apoptosis, that maintain tissue homeostasis of the colon. Malfunctions in these processes can lead to excessive cell numbers and formation of tumors. At the molecular level, the canonical Wnt/β-catenin signaling pathway, a major pathway in cell proliferation, is a key initiator of cell division. APC is a key component of the Wnt/β-catenin signaling pathway and plays a major role in the regulation of the pathway.

Colon Cancer research is progressing rapidly and produces a lot of biological information every year. An integrated analysis of this scattered biological information is very much needed so as to uncover the mechanisms behind the biological processes that lead to the initiation as well as the progression of colon cancer. This is where the role of theoretical thinking and modeling come in. Computational modeling is a powerful tool to integrate and make sense of biological information to generate new ideas and new hypotheses that can then be tested in the laboratory.

The intestinal epithelium renews itself every 4-6 days by a coordinated series of cell proliferation, migration and differentiation events. These events are initiated in the crypts of Lieberkühn. At the bottom of the crypt, there is a stem cell niche, where a small population of stem cells divide continuously, producing transit cells. These semi-differentiated transit cells represent precursors at different stages of commitment and have the ability to divide rapidly a limited number of times, after which they undergo terminal differentiation. At the same time, as new cells are being produced continuously, the mitotic pressure makes the entire population of semi- and terminally-differentiated cells migrate towards the luminal orifice where they are shed from the luminal surface. Approximately 95% of the time a stem cell divides asymmetrically to yield one stem cell daughter that remains in the niche and one transit daughter that

leaves the niche and differentiates. There is another mode of stem cell division , namely symmetric division that produces either two differentiated daughter cells or two stem cell daughters. When symmetric division produces two differentiated daughter cells, both the daughters leave the niche and that stem cell lineage becomes extinct in the niche. The reduction in stem cell number as a consequence of both daughters leaving the niche, is balanced by a self-renewing symmetric stem cell division that yields two stem cell daughters both of which remain in the niche. Eventually the symmetric stem cell divisions result in the periodic loss of all stem cell lineages except one - this is called "niche succession". This random, population drift type mechanism occurs periodically in all niches and does not require mutation or selection.

The tumor suppressor protein APC, has been known to play a decisive role in maintaining the fidelity of all the above processes that maintain colon crypt homeostasis. In this thesis we present the details of our investigation into the multiple ways in which mutations of the APC gene can disrupt the homeostasis.

Our investigation involves development of computational models and computational experiments using these models. We integrate into computational models, the biological information available on the multiple roles played by APC in maintaining the integrity of the colon crypt and the consequences of functional losses of APC on the overall functioning of the crypt. These models are then utilized as tools for performing computational experiments for studying the multiple ways in which APC mutations can lead to initiation and development of colon cancer. Computational experiments are powerful tools for in-depth understanding of processes which are governed by multiple phenomena. With these tools one can perform experiments that are impossible in reality like shutting different phenomena off and on, evolving and testing different hypotheses and asking "what-if questions".

We develop models at two different scales:

1. At the scale of the epithelial tissue covering the colon crypt, we develop Agent Based models where the cells constituting the tissue are considered as "Agents" that choose between various "Agent Actions" like division, differentiation, migration, or death depending on cues from their external environment as well as from their internal state of being, which is described by a set of "Agent

Attributes". The state of being keeps changing depending on the actions they choose to perform. Choice of action at any point of time is governed by stochastic rules. Definition of relevant Agent Attributes, Agent Actions and Rules are based on available biological information. These models thus enable effective integration of biological information of various kinds and the simulations can reveal overall consequences that emerge out of the integrated information. Chapters 3, 4 and 6 present tissue level models and computational studies using these models.

2. At the scale of molecular networks within a single cell we study the dynamics of the Wnt/β-catenin signaling pathway and the multiple roles APC plays in regulating this pathway. The Wnt pathway, when activated by Wnt signaling molecules, initiates cell division. Mutation of APC disrupts the regulation and causes continuous activation of the Wnt pathway even in the absence of Wnt signals, thus causing uncontrolled cell proliferation. Essentially APC regulates the Wnt pathway by controlling the formation of the nuclear complex β-catenin/TCF that initiates the transcription of the Wnt target genes. Integration of different ways by which APC may regulate the formation of these nuclear β-catenin/TCF complexes, we develop of a stochastic simulation model that is solved using the Gillespie algorithm. The model serves as a logical framework to untangle complex interactions and to demonstrate the possible outcomes from different hypotheses. This model and insights gained from simulations using the model are presented in Chapter 5. These insights are further used in extending the scope of the tissue scale model presented in Chapter 6.

AIM AND OBJECTIVES

The aim of this work is to understand how the APC protein, through its involvement in several different molecular and cellular processes, contributes to the healthy maintenance of the colonic epithelium and how mutations in the APC gene perturb these processes and contribute to the initiation of colon cancer.

The objectives are:

1. Development of a tissue level model of the normal colon crypt and study of the phenomenon of niche succession using the model.

   An agent-based computational model to simulate the dynamics of a normal crypt is built with cells of the colon crypt as agents. The model simulates the normal cellular processes in the crypt and the phenomenon of niche succession that occurs in normal crypts.

2. Extension of the crypt model to explain the pre-cancerous changes observed in Familial Adenomatous Polyposis (FAP) crypts.

   The normal crypt model is extended to include APC mutations and this leads to the postulation of a hypothesis that explains several experimentally observed feature of FAP crypts.

3. A molecular level understanding of the multiple roles of APC gene in the regulation of Wnt/β-catenin signaling pathway.

   A stochastic simulation model that incorporates five different mechanisms, by which APC can regulate the formation of the β-catenin/TCF complexes, is built to understand the multiple roles played by it in the regulation of Wnt/β-catenin signaling pathway.

4. Development of a crypt model for understanding the multiple roles of APC in the initiation of colon cancer.

   The normal crypt model is further extended to study the multiple ways in which APC mutations perturb the processes that maintain homeostasis of the crypt and identification of those perturbations that are critical to tumorigenesis and progression to cancer.

OUTLINE OF THE THESIS

An overview of the available biological information on the multiple roles played by APC gene in initiation and development of colon cancer is given in Chapter 1.

Chapter 2 gives an introduction to the two main computational techniques that are used in the development of the models in this work - the Agent Based Modeling and the Gillespie's Stochastic Simulation Algorithm. An overview of the computational models developed for colon crypt dynamics is also presented.

Chapter 3 presents the details of our tissue level model for the normal crypt dynamics and how the model can reproduce experimentally observed features of the colon crypt including the interesting phenomenon of niche succession.

In Chapter 4 we apply the model for colon crypt dynamics and niche succession to evolve and test a hypothesis on how a heterozygous APC mutation can produce the experimentally observed features of an FAP crypt.

In Chapter 5 we present a molecular network level model of Wnt signaling that includes multiple ways in which APC can regulate the Wnt/β-catenin signaling pathway and how APC mutations can affect the fidelity of the molecular regulation processes.

In Chapter 6 we further extend our model for crypt dynamics to explicitly include multiple roles played by APC in controlling the tissue level behavior of mutated and unmutated cells.

Chapter 7 summarizes the different outcomes of the work and outlines directions for continuation of this research.

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LIST OF ABBREVIATIONS

TA	Transit Amplifying
APC	Adenomatous Polyposis Coli
CRC	Colorectal Cancer
Wnt	Wnt Ligands
TCF	Transcription Factor
MCC	Mutated in colorectal cancer
TP53	Tumor protein p53
kDa	Kilo Daltons
cDNA	Complementary DNA
kb	Kilo-Base Pairs
bp	Base Pair
GC	Guanine-Cytosine
PP2A	Protein phosphatase 2
ASEF	APC-Stimulated Guanine Nucleotide Exchange Factor
GEF	Guanine Nucleotide Exchange Factor
Cdc42	Cell Division Cycle 42
KAP3	Kinesin Superfamily Associated Protein
IQGAP1	IQ motif Containing GTPase-Activating Protein 1
AMER	APC Membrane Recruitment Protein
MCR	Mutation Cluster Region
NES	Nuclear Export Sequence
CRM-1	Chromosome Region Maintenance 1
aa	Amino Acid
CID	Catenin Inhibitory Domain
CtBP	C-terminal Binding Protein
GSK3β	Glycogen Synthase Kinase 3β
SH3	Src Homology 3
ArfGAP	ADP-Ribosylation Factor GTPase-Activating Protein
DDEF	Development and Differentiation Enhancing Factor

NLS	Nuclear Localization Sequence
Pol-β	DNA Polymerase β
BER	Base Excision Repair
S/T	Serine/Threonine
EB1	End Binding Protein
PDZ	Psd-95 (Post Synaptic Density Protein), DlgA (Drosophila Disc Large Tumor Suppressor) and ZO1 (Zonula Occludens-1 Protein)
hDLG	Human Homolog of the Drosophila Discs Large Tumor Suppressor protein
CK1α	Casein Kinase 1-α
KIF	Kinesin Superfamily Proteins
DlG	Drosophila Melanogaster Discs Large Protein
CLIP	Cytoplasmic Linker Protein
Nup	Nucleoporin
ES cells	Embryonic Stem Cells
BUB1B	Mitotic Checkpoint Serine/Threonine Kinase B (Budding Uninhibited by Benzimidazoles 1)
MAD2L1	Mitotic Arrest Deficient 2 Like 1
CIN	Chromosomal Instability
kMAP	Kinetochore-Bound MT-Associated Protein
N-APC	N-terminal APC Fragments
LP-BER	Long Patch Base Excision Repair
SP-BER	Short Patch Base Excision Repair
Fen-1	Flap Endonuclease 1
DRI domain	DNA Repair Inhibitory Domain
DSB repair	Double Stranded Break Repair
VPA	Valproic Acid
HDAC	Histone Deacetylases
ROS	Reactive Oxygen Species
FAP	Familial Adenomatous Polyposis
AFAP	Attenuated Form of FAP
β-TrCP	β-transducin Repeat-Containing Protein

CRD	Cysteine Rich Domain
FZD	Frizzled
LRP	Low-Density Lipoprotein
Dsh/Dvl	Dishevelled
LEF	Lymphoid Enhancer Factor
ABM	Agent-Based Model
SSA	Stochastic Simulation Algorithm
EBM	Equation-Based Model
ODE	Ordinary Differential Equation
PDE	Partial Differential Equation
SDE	Stochastic Differential Equation

LIST OF ABBREVIATIONS, SYMBOLS AND PARAMETERS USED IN THE MODEL

N	Number of columns
M	Number of rows
N_0	Initial number of Stem Cells
TC_s	Cell Cycle Time for Stem Cells
TC_t	Cell Cycle Time for Transit Cells
$TG1_s$	Time in G1 State by Stem Cells
$TG1_t$	Time in G1 state by Transit Cells
Num_div_max	Maximum number of divisions before terminal differentiation
Δt	Time step
y_0	Position of Stem Cell niche
y_d	Starting position of the Wnt free zone
APC_{conc}	The amount of APC protein expressed within a cell
Wnt	Concentration of Wnt Signaling molecules in the environment of the cell
P_{wnt}	Probability of the transition from the quiescent state to the cell cycle
P_s	Symmetric division probability
P_d	Differentiation probability
P_0	Probability that no stem cells are produced
P_1	Probability that one stem cell is produced
P_2	Probability that two stem cells are produced
B	Biasing factor
Mutation State1	Mutation state of alleles of the cell agent
Mutation State2	Mutation state of alleles of the cell agent
Mutation probability	Probability for second mutation
Axin/APC	Axin/APC Complex

D	Destruction Complex
BD	β-catenin bound to D
CK1-pBD	CK1 Phosphorylated β-catenin bound to D
GSK3-pBD	GSk3β Phosphorylated β-catenin bound to D
GSK3-pB/ID	GSk3β Phosphorylated β-catenin bound to Inactive D
ID	Inactive Destruction Complex
ABC	Active β-catenin (β-catenin Phosphorylated at Serine 45)
pABC	ABC Phosphorylated at Y142
WC	Wnt Receptor Complex
WD	Destruction Complex bound to Wnt
WBD	β-catenin bound to WD
CK1-WBD	Phosphorylated β-catenin bound to WD
GSK3-WBD	GSk3β Phosphorylated β-catenin bound to WD
Axin/B-Cat	β-catenin bound to Axin
APC/B-Cat	β-catenin bound to APC
Mutation	Level of functional APC expressed in the cell
P	Probability of entering cell cycle with contact inhibition included.
Occupancy of the grid	Number of cells occupying the same grid location
K	Proportionality constant
Maximum Occupancy	Number of cells that can remain in a grid without pushing
P_shedding	Probability of Shedding

1

APC and Colon Cancer

This chapter gives an overview of the multiple roles of the Adenomatous Polyposis Coli (APC) gene in the maintenance of the colon epithelium and how mutations in APC can initiate colon cancer.

1.1 The colon

The colon (also known as large intestine or large bowel) is the terminal part of the lower gastrointestinal tract which is about 5 feet long and 3 inches in diameter. The major function of the colon is to absorb water, salt and nutrients from solid waste and process waste products from the body to prepare it for elimination. The colon is lined by a single layer of epithelial cells that is renewed every 3-5 [1] days through continuous proliferation, migration, differentiation and apoptosis. Malfunctions in this series of processes may result in cancer of the colon.

Underneath this single layer of epithelial cells lies the lamina propria which contains the connective tissue and immune cells. A combination of the epithelium and the lamina propria is referred to as the mucosa or mucous membrane. The monolayer of epithelial cells invaginates into the lamina propria and these finger-like invaginations are termed the proliferative tubular pits or crypts. The human colon is subdivided into about 2×10^7 such crypts. Each crypt is about 75 - 110 cells long with around 2000 cells and an average crypt circumference of 23 cells [2].

The base of the crypt is a stem cell niche, composed of subepithelial intestinal myofibroblasts, where the self renewing Intestinal Stem Cells (ISCs) reside. These stem cells are capable of regenerating all intestinal cell types. In a human colonic crypt, there are around 5–10 functional ISCs [3, 4]. The stem cells in the niche proliferate and give rise to the cells that line the colonic crypt. When new cells are formed the old cells in the proliferative compartment move upwards in the colon through directional migration

towards the crypt collar. This is considered a passive cell movement due to the pressure of mitotic activity below. Crypt cells can move rapidly and can cover up to 50 μm in 12 h [5]. The dividing daughter cells of the stem cells called the Transit Amplifying (TA) cells or progenitor cells are found in the lower two-third of the crypt. TA cells undergo around 4–6 rounds of rapid cell division [6] after which they get fully differentiated. The fully differentiated cells (colonocytes, goblet cells, enteroendocrine cells) occupy the upper third of the crypt. The fully differentiated cells are non-dividing or quiescent cells. Figure 1.1(A) shows the organization of the colonic crypt. When cells reach the crypt collar they lose contact with the underlying basement membrane and are finally shed into the intestinal lumen [7, 8]. Due to this high turnover rate in the intestinal epithelium there is a high probability of tumor development and growth which can lead to cancer. At the same time, the frequent and regular renewal of cells ensures that cells carrying mutations do not live long enough to interrupt tissue homeostasis. Therefore the long-lived ISCs in the stem cell niche are believed to be the cells of origin for tumorigenesis [9].

Two essential characteristics of adult tissue stem cells are “longevity” (they often live through the entire lifetime of the organism) and “multipotency” (ability to generate the multiple differentiated cell types of the tissue)[10]. As per the Unitarian theory, all the different cell types of the crypt are derived from a single stem cell [11]. When a cell leaves the stem cell niche, it becomes less stem-cell-like and more differentiated [12, 13].

Mostly stem cells undergo asymmetric cell division, giving rise to one stem cell and one daughter cell committed for differentiation [14](Figure 1.1(B) a). The stem cell remains in the niche whereas the daughter cell leaves the niche. The tumor suppressor gene, Adenomatous Polyposis Coli (APC), plays significant roles in regulating the orientation of mitotic spindles perpendicular to the stem cell niche and favoring asymmetric stem cell division [15]. But stem cells also undergo symmetric cell division producing either two stem cells that remain in the niche (Figure 1.1(B) b) or two daughter cells that leave the niche(Figure 1.1(B) c). This may compensate for any random gain or loss of a stem cell[16]. A balance between symmetric and asymmetric stem cell divisions is critical in order to maintain a constant stem cell number and its imbalance is implicated in colon cancer.

When a stem cell divides symmetrically, resulting in two daughter cells that leave the niche, that stem cell lineage becomes extinct. In order to maintain a constant stem cell number in the niche, this loss has to be compensated by a symmetric stem cell division that results in two stem cells that remain in the niche. Eventually all stem cell lineages except one become extinct and that lineage will come to dominate the niche. This random process of niche succession is termed 'neutral drift'. In the human colon, niche succession happens every 8.2 years [17]. Descendants of a stem cell lineage that carries proliferative mutations may take over the whole crypt due to selection and this process is termed clonal conversion [18]. In adults every crypt is said to be monoclonal whereas, in the fetus and neonates, the crypts are polyclonal [19].

Figure 1.1(C) shows the phenomenon of niche succession: (a) In the stem cell niche, (b) a stem cell gets mutated (blue)(c) and a clone of mutated cells is developed from this mutant stem cell in the dividing transit zone through asymmetric division (d) if the clone is not lost by apoptosis or stem cell division producing two TA cells, this mutant stem cell may expand through symmetric divisions that gives two stem cell progenies and colonize the niche (e) and the lineages from the mutant stem cell dominates the niche. Figure 1.1(D) shows clonal conversion: the crypt is replaced by descendants of a mutated stem cell.

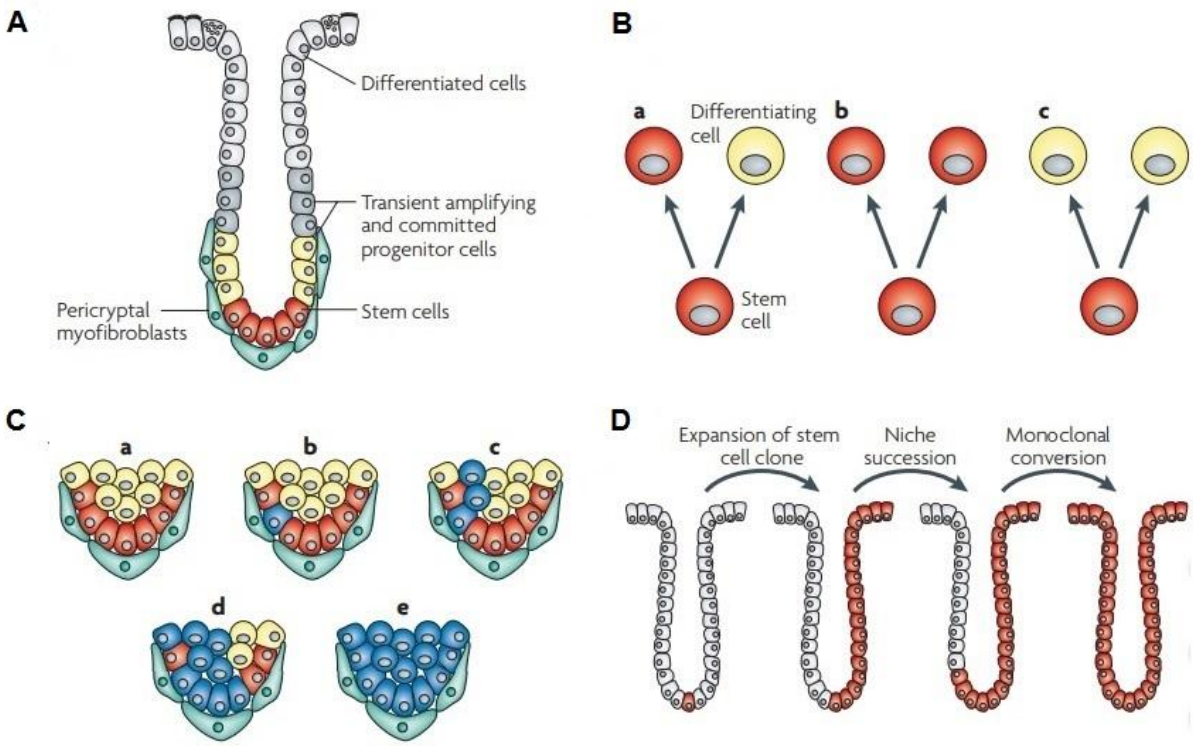


Figure 1.1 Colonic crypt organization, asymmetric and symmetric stem cell divisions, niche succession and clonal conversion. (A) Colonic crypt organization (B) Asymmetric and symmetric stem cell divisions, (C) Niche succession (D) Clonal conversion. (Reproduced from Adam and Nicholas (2008) [20].

1.2 Colon cancer

The cancer of the colon or the rectum is known as colorectal cancer and can be named as colon cancer or the rectal cancer depending on the organ from which it develops. The colon cancer and rectal cancer are grouped together as they have many features in common and called 'the colorectal cancer (CRC)'. Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world. CRC is the second most common cancer in females and third most common cancer in males globally with estimated numbers of around 1.4 million new cases per year [21]. CRC is a deadly disease with a 5-year survival rate of less than 65% [22].

Cancer is a group of diseases characterized by an abnormal cell growth. When abnormal growth of tissue (neoplasm), forms a mass, it is referred to as a tumor. A tumor can be either benign (non-cancerous) or malignant (cancerous). Prior to the abnormal growth of tissue as neoplasia, the cells go through abnormal changes called hyperplasia and dysplasia. In hyperplasia, there is an increase in the number of cells and sometimes the

size of the cells also increases in an organ or tissue, but the cells appear normal. In dysplasia there is disordered growth or maturation of the tissue with greater variability between cells. Hyperplasia and dysplasia are not cancerous but may be a stage on the way to cancer development.

Colorectal cancer develops very slowly and mostly takes more than a period of around 10 to 15 years [23]. CRC begins as protrusions or abnormal growth of tissue on the inner lining of the colon or rectum known as 'polyps'. Usually, polyps arise from the mucosal layer of the colon or rectum. Colorectal polyps may be histologically classified as neoplastic and non-neoplastic polyps. The neoplastic (adenomatous) polyps are benign tumors that develop from the mucus-secreting colonic epithelial cells and have the potential to develop into cancer. Adenomatous polyps or adenomas are hence considered pre-cancerous. Early diagnosis and removal of these polyps can prevent the disease. Adenomatous polyps are further classified histologically into tubular, villous, or tubulo-villous adenomas [24, 25]. 65–80% of adenomatous polyps are tubular adenomas and appear like branched tubular glands. 5–10% of adenomatous polyps are villous adenomas having long, finger-like projections. Tubulo-villous adenomas account for around 10–25% of polyps and have both tubular and villous growth patterns. All types of adenomas exhibit some degree of dysplasia and can be classified into three degrees of severity: mild, moderate and severe, and the grade of severity can be determined by architectural features like crypt branching and budding, or complex glandular crowding and cytological features like loss of cell polarity and differentiation or enlarged and rounded nuclei with nucleoli. Risk of malignancy increases with increase in the degree of dysplasia. Carcinomas (cancer that develops in the epithelial tissue) exhibit high-grade (severe) dysplasia. The degree of dysplasia is usually associated with the size of the polyp [26, 27]. Even though adenomas have the potential to develop into cancer, only less than 10% of adenomas will evolve to become adenocarcinomas. Non-neoplastic polyps are not malignant and are grouped into hyperplastic polyps, hamartomas polyps, lymphoid aggregates and inflammatory polyps [28-30].

Familial Adenomatous Polyposis (FAP) was identified as a heritable pathogenic syndrome in the 1920s and was first reported in literature as a disease with dominant inheritance pattern in 1925 [31]. This second most common predisposing genetic syndrome is characterized by development of multiple polyps in the colon and rectum at an early age of around 10 -12 years. The number and size of the polyps increase with age. By around 20 years of age, cancer develop in one or more of these polyps and the life time risk of CRC by an average age of 40 is nearly 100% if left untreated, and by the time the colon will be occupied by hundreds to thousands of colonic adenomas [32-34]. FAP is an autosomal dominant genetic disease and accounts for less than 1% of all CRCs. FAP is usually caused by germline or inherited mutations in the APC gene on chromosome 5q21–q22.

Other Syndromes that are caused by a germline mutation in the APC gene and come within the FAP spectrum include Gardner syndrome, Turcot syndrome, and attenuated form of FAP (AFAP). Gardner syndrome is characterized by colorectal adenomas, osteomas or bony growth most commonly on the skull and the mandible, dental abnormalities, desmoid tumors and epidermoid cysts. Turcot syndrome is characterized by colorectal adenomas and medulloblastoma, tumors of the central nervous system. AFAP is a milder form of FAP characterized by fewer colonic polyps than in classic FAP with around 30-35 located in the proximal colonic area with a lifetime risk of developing CRC of around 70% [35-38].

1.3 APC involvement in colon cancer

Colorectal cancers develop through a series of orderly events beginning with a normal colonic epithelium altered to an adenomatous intermediate and then finally the adenocarcinoma, the so-called “adenoma-carcinoma sequence” [39]. Fearon and Vogelstein [40] first proposed a multistep genetic model (Vogelstein's model) that explains the orderly mutations that leads to the events in the transition from normal colonic epithelia via increasingly dysplastic adenoma to malignant cancer. According to Fearon and Vogelstein the events in carcinogenesis is initiated with the accumulation of mutations in the APC gene, followed by activating mutations of K-ras, the oncogene and inactivation of TP53, the tumor suppressor gene (Figure 1.2).

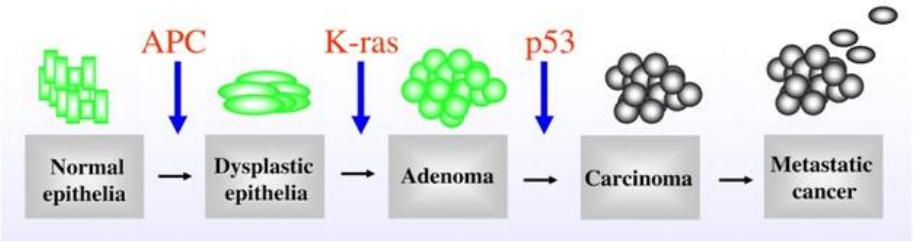


Figure 1.2 Schematic illustration of the order of mutations in the progression of events from normal epithelia to metastatic cancer. (Reproduced from Gillian Smith et al. (2002)) [41].

Aberrant crypt foci (ACF) are clusters of abnormal tube-like glands formed in the lining of the colon which precede adenomas and are thought to be the earliest identifiable abnormality in colon crypts that appear to be abnormal. Dysplastic ACF, that harbor mutations in APC gene exhibit the highest malignant potential [42, 43]. It is apparent that APC gene plays important roles in the initiation of CRC [44] and APC mutations are said to be sufficient for the growth of early colorectal adenomas [45].

The most well known involvement of APC in colon cancer is through its participation in the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is the predominant signaling pathway that regulates proliferation and the maintenance of the intestinal stem cells and elevated Wnt signaling is highly implicated in colorectal cancer. APC is a key regulatory component of the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway is detailed in Section 1.5.

At the crypt base, the stem cells are surrounded by mesenchymal cells, the pericryptal myofibroblasts, that produce the Wnt signaling ligands that bind to Wnt receptors to activate the Wnt/β-catenin signaling pathway [20]. Wnt signaling in crypt cells has been observed to be highest at the crypt base and keeps decreasing along the crypt axis [46]. Position of stem cells is in close proximity to the source of Wnt ligands at the crypt base. As they diffuse upward, Wnt exposure decreases for TA and differentiated cells, which lead to growth arrest and differentiation.

Concentration of APC has been observed to be low at the crypt base and keeps increasing towards the top of the crypt. Since WNT and APC gradients are counter to each other a counter-current-like mechanism was proposed which sets a zone in the lower crypt with most favorable conditions for maximal cell division [47]. Increased levels of Wnt signaling as a result of mutations in APC are also highly implicated in colon

cancer cases. APC mutation activates the Wnt/β-catenin signaling pathway by stabilization of the transcriptional co-activator β-catenin, leading to its nuclear translocation and its binding with transcriptional factors(TCF) resulting in gene expression of proliferative genes such as *c-myc, cyclin D1* etc [48] leading to malfunctions like increased proliferation, failure to differentiate and also aberrant cell migration[49].

It is believed that any cell in the mucosa can acquire and accumulate genetic mutations and eventually lead to malignant transformation and this is termed as "the somatic" mutation theory of cancer [50]. APC has been described as a genetic gatekeeper that is required for maintaining tissue homeostasis in colonic epithelium and is found to play significant roles in the orderly events like cell proliferation, differentiation, migration and apoptosis that maintain the tissue homeostasis of colonic epithelium. Truncating APC mutations resulting in any imbalance in these orderly events, induce polyp formation [51]. Mutations in APC are highly implicated as the initiating event in both sporadic as well as inherited germline colon cancer cases.

Crypts of FAP patients (patients with germline or inherited APC mutations) show an increased ability to divide. The mitotic distribution was found to reach a highest point at positions around 40–50% along the crypt axis of healthy crypts. An expansion of the proliferative zone was observed in crypts that harbor APC mutations [52, 53]. The bifurcation of a crypt to produce two daughter crypts is termed crypt fission. Regular crypt replacements happen through crypt fission in normal human colonic crypts. Increased crypt fission is observed in FAP crypts. It has been proposed that the size of the crypt and the size of the crypt stem cell population may be the regulating factors of crypt fission [54, 55]. The shift in the distribution of proliferative cells along the crypt axis [56-59] and also increased crypt fission [60, 61] observed in FAP colons indicates an increase in stem cell number. Hence APC mutations may be contributing to increase in stem cell number[62].

1.4 The APC gene and protein

The first hint as to the location of the gene that caused Familial Adenomatous Polyposis came from Herrera et.al [63] in 1986. A man with FAP was found to have a region missing from one of his two copies of chromosome 5. Thus the occurrence of FAP was

found to be linked with the q21-q22 region of chromosome 5 [64]. Almost to the end of 1980's studies revealed a region on the long arm of chromosome 5, which included APC and MCC genes separated from each other by 150 kbp. Identification of the APC gene was supported by the observation that two patients harbored deletions at the 5q21 locus that spanned 100 kb of DNA. In 1991 the three candidate genes, DP1, SRP19 and DP2.5, found in this critical region, were examined in FAP patients for mutations that could be involved in APC. The DP2.5 gene has sustained four distinct mutations specific to APC patients resulting in premature stop codons from which one was transmitted from generation to generation indicating this to be the APC gene [65].

1.4.1 The APC gene structure

Adenomatous polyposis coli (APC) also known as Deleted in Polyposis 2.5 (DP2.5) is a multi-functional tumor suppressor protein and is encoded by the APC gene. APC is widely expressed in a variety of tissues, especially throughout the large intestine and central nervous system during both embryonic and postnatal periods.

The APC gene was identified and cloned by two groups, simultaneously and individually in 1991 and a 2843 residue protein with a molecular mass of 311.8 kDa was deduced. The deduced protein sequence did not contain nuclear targeting signals or transmembrane regions, suggesting cytoplasmic localization of APC. In 1991, it was predicted that the APC gene product contain coiled-coil regions and was expressed in a wide variety of tissues and also, a 10 kb APC mRNA was identified by Northern blot analysis and found that APC gene contains 15 exons [65-68].

In 1993, cDNA clones that represent transcripts expressed in human fetal brain, that code for 5-prime end of APC gene was isolated. Identification of alternative 5-prime untranslated region comprising at least 103bp from sequence analyses suggested that two APC-specific promoter elements exist, giving rise to two different untranslated regions. 3 additional AUG codons, located 5-prime to the intrinsic APC initiation site within the alternative untranslated regions was identified suggesting that these codons may be significant for the translational regulation of APC gene expression [69].

In 1993, a study conducted in transcripts from human colorectal tumor cell lines suggested the presence of mutations in the transcriptional control region of APC and reported at least 5 different forms of 5-prime noncoding sequences [70]. In 1995, a new

coding exon (exon 10A) of the APC gene was identified which is 54 nucleotides long, located 1.6 kb downstream from exon 10 and is the subject of alternative splicing that when transcribed, adds an additional 18 amino acids to the APC protein. This highly conserved exon is expressed in a tissue-independent manner [71]. In the same year, an evolutionarily conserved exon between the known exons 10 and 11 that contains a heptad repeat motif was also identified [72]. In 2005, Karagianni et al. [73], identified an untranslated exon (exon N) within which a promoter region holds features of a housekeeping gene, including high average GC content and strong promoter activity.

APC gene contains 8538 bp spanning 21 exons which are within a 98-kb locus [74]. Exon 15 is very long with 6579 bp open reading frame, and accounts for more than 75% of the coding sequence and is the most common target for both germline and somatic mutations[75]. The 8538 bp open reading frame of APC gene codes for a protein of 2861 amino acids. Exon 10A is the smallest exon. The transcript of APC gene that occur in abundance lacks exon 10A and has a mass of 310 kDa, comprising 2843 amino acids [76, 77]. Seven exons of the APC gene are alternatively expressed and encode alternate isoforms of APC protein with different tissue-specific patterns. The molecular weight of these proteins range from 90 to 310 kDa and differ in their amino termini and their capabilities for dimerization [70, 76, 78].

1.4.2 The functional domains of the APC protein

APC has multiple domains that bind to different proteins to carry out multiple functions (Figure 1.3). The large 311kDa APC protein can be divided into N-terminal, Central and C-terminal regions. The N-terminal region in APC protein is predicted to be most stable and well-ordered region, while the central, and C-terminal regions are predicted to be flexible and disordered mostly [79].

1.4.2.1 The N-terminal region

The APC protein consists of an oligomerization domain followed by an armadillo repeat (Arm) domain in its N-terminus. The first 900 residues in APC protein contain proline-free blocks with a series of heptad repeats of hydrophobic residues with sequence homologies to coiled coils which may facilitate intra- and inter-molecular binding and is implicated in protein-protein interactions. These heptad repeats allow APC to form homo-dimers. Amino acids 6–57 in APC protein are essential for this oligomerization

and this region in the N-terminus is known as the oligomerization domain [80, 81]. In the oligomerization domain wild-type APC may form dimers with wild-type as well as truncated mutant APC proteins thereby decreasing the amount of available wild-type APC protein in addition to its decrease because of a mutant APC protein. Hence the oligomerization domain in APC provides a mechanism by which APC mutants may have a dominant negative effect in reducing the function of APC as a tumor suppressor [82-85].

The Arm domain in APC is well conserved and binds with a wide variety of proteins. Arm repeats are found to be embedded in the heptad repeats of the APC protein. Residues 452–767 was predicted to form seven 42aa repeats that form a right handed super helix (R1–R7) [86, 87]. Each repeat has three α helices - H1, H2 and H3. Repeat R1 does not have helices H1 and H2. The Arm repeats R2–R7 are homologous and take part in similar intra- and inter-repeat interactions. APC Arm is cylindrical in shape with an approximate length of 65A° and diameter of 33A°. The superhelix has an L-shaped groove which is large enough to accommodate a polypeptide chain [88]. The Arm domain binds several proteins including B56 regulatory subunit of protein phosphatase 2A (PP2A) which is a heterotrimeric complex implicated as a regulator of Wnt/β-catenin Signaling pathway [89], APC-stimulated guanine nucleotide exchange factor (ASEF) which is a guanine-nucleotide exchange factor (GEF) specific for Rac1 and Cdc42 via APC-binding region in the NH2 terminus of ASEF. ASEF was found to be involved in cell migration [90]. The Arm domain also binds kinesin superfamily-associated protein 3 (KAP3) which is a linker protein for kinesin motor proteins involved in cell migration [91], IQ-domain GTPase-activating protein 1 (IQGAP1) which was found to be involved in cell migration and cell adhesion [92], APC membrane recruitment 1 (AMER1), a plasma membrane-associated protein involved in the distribution of APC [93].

In Arm domain, amino acids 334 - 625 were found to be associated with nuclear localization [94]. Two nuclear export signals (NES) was also identified at the N-terminal region of APC at amino acids 68-77 and 165-174 that are required for shuttling of APC between the nucleus and cytoplasm and shuttling may be maintained through direct interaction of NES with exportin CRM-1 [95, 96].

1.4.2.2 The central region

The central region of the APC protein contains different types of repeats that are vital to its role in the negative regulation of Wnt/β-catenin signaling [97, 98] namely 15-amino acid (15aa) repeats, 20-amino acid (20aa) repeats, SAMP (serine-alanine-methionine-proline) repeats and the Catenin Inhibitory Domain (CID). There are four S/T rich 15aa repeats (15A, 15B, 15C, 15D) between residues 1020-1188. A crystal structure revealed that 15aa repeats bind in the positively charged long groove formed by armadillo repeats 5–8 of β-catenin [99]. Kohler et al. [100] reported that binding of β-catenin to the 15aa repeat of APC is necessary and sufficient to target β-catenin for degradation. Their studies also revealed that the first 15aa repeat is one target of the positive selection of mutations that lead to tumor development. APC binds to C-terminal binding protein (CtBP) through 15aa repeats. This results in the sequestration of the APC/β-catenin complex redirecting free nuclear β-catenin away from the Wnt transcription factor TCF-4 thereby inhibiting TCF mediated trascription in the nucleus [101].

There exist a series of seven repeats of 20aa (20R1, 20R2, 20R3, 20R4, 20R5, 20R6, 20R7) between residues 1264-2036 in APC protein that are required for β-catenin turn over. Each repeat is comprised of the amino acids TPXXFSXXXSL and can bind Glycogen Synthase Kinase 3β (GSK3β) to form binding sites for β-catenin and are known as the β-catenin binding repeats [65, 102]. These repeats have great similarity to one another and are highly conserved. Their binding affinity for β-catenin varies greatly suggesting different biological functions and shows dramatically increased affinity of 300 to 500 fold upon phosphorylation. The crystal structure reported by Yi Xing et al. [103] revealed that the phosphorylated 20aa repeat binds to the groove of armadillo repeats 1–5 of β-catenin, and multiple phosphorylated residues in 20aa repeat interact with β-catenin. Down-regulation of β-catenin is dependent on at least three of the seven 20aa repeats in APC [98]. 20R3 is shown to have the tightest β-catenin binding site and also 20R3 is lost in APC mutations in colon cancer suggesting its significant role in Wnt signaling and colon cancer. An important upstream, charged residue is lacking in 20R2 and hence it cannot bind β-catenin [104], but 20R2 is the only 20aa repeat required for APC2 function in the Destruction Complex [105].

Scattered amongst the 20aa repeats are three repeats of 31-32 amino acids between residues 1563 and 2057, each containing the Ser-Ala-Met-Pro sequence and are called

SAMP repeats (SAMP1, SAMP2, SAMP3). SAMP repeats are axin binding regions which also bind to conductin or axin1, a homolog of Axin [106, 107]. Axin binding is mapped to a region containing the conserved motif I/L-L-X-X-C-I-X-S-A-M-P which contain the hydrophobic motif and the downstream SAMP motif [106, 108]. SAMP repeats are found to specifically bind Src homology 3 (SH3) domains of development and differentiation enhancing factor (DDEF), which is an ADP-ribosylation factor (Arf) GTPase-activating protein (GAP (ArfGAP)) involved in the regulation of focal adhesions giving additional evidence for the role of APC in cell migration [109]. Most of the APC mutations generate APC truncations typically missing some of the 20Rs and all of the SAMP repeats [75] .

Kohler et al. [110] revealed a new APC domain, the β-catenin inhibitory domain (CID) spanning residues 1404 to 1466 located between the 20R2 and 20R3 and is found in many truncated APC products. In truncated APC, the CID is very much necessary in maintaining β-catenin turn over and down-regulation of transcriptional activity.

In 1999, Deka et al. [111] mapped one DNA binding domain in the C-terminal domain of APC which contain clusters of repetitive S(T)PXX sequence motifs spanning residues 1340 – 1901. In 2000, Rosin-Arbesfeld et al. [112] gave evidence that APC contains highly conserved three nuclear export signals (NESs) in the central domain of APC spanning residues 1506-1511, 1657-1662, and 2027-2032 located immediately downstream the mutation cluster region. In the same year F. Zhang et al. [113] identified two potential Nuclear Localization Sequences (NLSs) spanning residues 1767–1772 and 2048–2053 in the C-terminal domain of APC. R. Balusu et al. [114] in 2007, mapped the APC interaction site in DNA Polymerase β (Pol-β), an enzyme in the base excision repair (BER) pathway spanning residues 1245-1273 and found that Thr79, Lys81, and Arg83 of Pol-β were critical for its interaction with APC.

1.4.2.3 The C-terminal region

The C-terminal region of APC protein contains a basic domain that lies between amino acids 2200 and 2400 [65] which contain a large proportion of residues arginine, lysine and a good amount of proline that can bind microtubules and also actin [115, 116]. The C-terminal 170 amino acids can bind to EB1, a small microtubule-end-binding protein. The C-terminal 15 residues form a binding site for PDZ domains [117]. APC's C-terminal

amino acids binds through its S/TXV sequence to the PDZ domain of human homolog of the Drosophila discs large tumor suppressor protein (hDLG) [118]. At the extreme C-terminus of APC, the VTSV motif binds PTP-BL, the protein tyrosine phosphatase through its PDZ domain [119]. Siah-1, the human homolog of Drosophila seven in absentia interacts with the C-terminal of APC and promotes degradation of β-catenin in response to p53 activation [120]. Deka et.al [111] mapped two DNA binding domains in the C-terminal third of the APC protein having clusters of repetitive S(T)PXX sequence motifs as in the β-catenin binding domain spanning residues 2219 – 2580 and 2581 – 2843 and are often found to be deleted in cancer. Mutations often result in the loss of C-terminal sequence of the APC protein which is essential for the initiation of colon cancer.

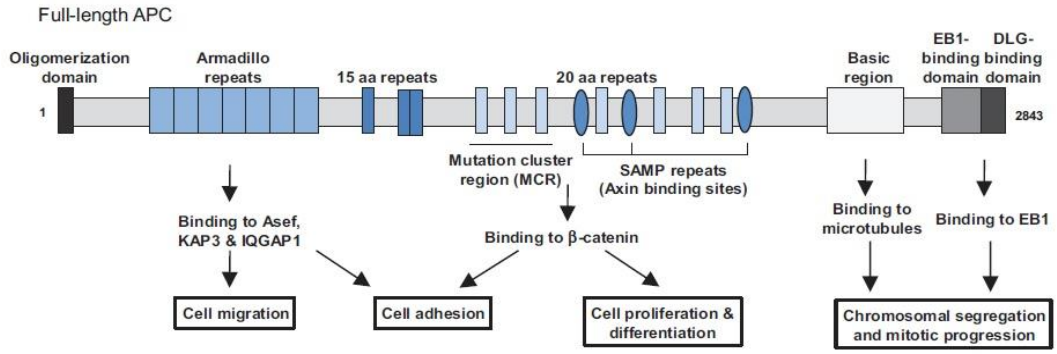


Figure 1.3 The functional domains of full length APC protein. (Reproduced from Zhang and Jerry (2017)) [121].

1.4.3 Functions of APC

The tumor suppressor, APC as a dynamic protein that shuttles between the cytoplasm, the cell periphery and the nucleus, and also as a protein with multiple binding domains, contribute to a wide range of protein functions and directly or indirectly regulate various cellular processes. APC is found to play critical roles in Wnt/β-catenin signaling, cell adhesion, cell migration, cell polarization, chromosome instability, cell cycle regulation, DNA repair, apoptosis etc. Role of APC as a tumor suppressor is well-known in colon cancer. Through both loss of tumor suppressive function and gain of tumor suppressive function APC plays critical roles in the initiation and progression of colon cancer.

1.4.3.1 APC in Wnt/β-catenin signaling pathway

The first identified function of APC was its role in the Wnt/β-catenin signaling pathway, which is a major pathway in cell proliferation. APC plays a significant role as a negative regulator of the Wnt/β-catenin signaling pathway by controlling the levels of cellular β-catenin, which when enters the nucleus, trigger the overexpression of oncoproteins resulting in increased proliferation and decreased differentiation, consistent with the reported expansion of the proliferative zone along the crypt axis [56-59]. It was reported that loss of APC through activation of Wnt/β-catenin signaling perturbs differentiation, migration, proliferation, and apoptosis [49]. Under normal cellular conditions when there is no Wnt signal, β-catenin levels are maintained at low levels in the cytoplasm as well as in the nucleus by its degradation by a Destruction Complex with APC, Axin, Glycogen Synthase Kinase 3-β (GSK3β) and CK1α (Casein Kinase 1-α). Phosphorylation of β-catenin at specific residues by the Destruction Complex marks β-catenin for ubiquitination and subsequent degradation by the proteasome machinery. But Wnt stimulation inhibits the activity of the Destruction Complex resulting in accumulation of β-catenin in the cytoplasm as well as in the nucleus. Nuclear β-catenin acts as a cofactor and interacts with transcription factors to mediate Wnt target gene expression (The Wnt/β-catenin signaling pathway is detailed in Section 1.5).

In 1993, APC was found to be associated with β-catenin and α-catenin [122, 123]. In 1995, the central region of the APC protein, which is found to be deleted or truncated in tumors, was identified to be responsible for the down-regulation of β-catenin [97]. GSK3β and Axin were also identified as negative regulators of β-catenin stability along with APC [124-127]. Studies in Xenopus egg extracts have demonstrated that APC, Axin and GSK3β are essential for degradation of β-catenin[128].

In 1996, APC was identified as a good substrate for GSK3β and the phosphorylation sites were mapped to the central domain of APC. It was also reported that when β-catenin is present in excess, APC binds to GSK3β. Binding of β-catenin to the phosphorylation sites of APC was found to be dependent on phosphorylation by GSK3β [102]. APC acts as a scaffold bringing together β-catenin and the other components of the Destruction Complex that phosphorylates and degrades β-catenin thereby inhibiting β-catenin/TCF dependent transcription. APC was suggested to simultaneously bind to Axin and β-catenin to promote efficient GSK3β phosphorylation of β-catenin.

Phosphorylation of 15aa and 20aa repeats of APC by GSK3β and CKIα was found to increase its affinity for β-catenin. Mutations in APC that prevent GSK3β-mediated phosphorylation and β-catenin degradation result in β-catenin pooling [129-131]. Studies by Homma et al. [132] reported association of APC with the α and β subunits of Casein kinase-2 (CK2). APC was found to bind to Axin through SAMP repeats. Kunttas-Tatli et al. [133] suggested that APC plays significant role in the negative regulation of Wnt β-catenin signaling pathway through SAMP repeats which mediate binding of APC and Axin. Over expression of the central domain of APC with 15aa and 20aa repeats and SAMP repeats was found to be sufficient to bind to, and to downregulate β-catenin [97, 106, 107]. It was reported that after phosphorylation of β-catenin bound to Axin, the 20aa repeats of APC gets phosphorylated, increasing its affinity for β-catenin over that of Axin. β-catenin therefore binds to APC leaving Axin which is free to bind another β-catenin[134, 135].

In the year 2000, Neufeld et al. [136] revealed two intrinsic leucine-rich nuclear export signals (NESs) near the amino terminus that mediate the export of APC. Each nuclear export signal could induce the nuclear export of a fused carrier protein. Mutation of both nuclear export signals of APC resulted in the nuclear accumulation of the full-length APC protein of approximately 320 kDa. From these findings it was suggested that APC is a nucleocytoplasmic shuttling protein whose cytoplasmic localization requires nuclear export signals, and hence APC may be important for signaling between the cytoplasmic and nuclear compartments of epithelial cells. In the same year Rosin-Arbesfeld et al. [112] gave evidence that APC contains highly conserved three nuclear export signals in the central domain of APC. F. Zhang et al. [113] identified two potential Nuclear Localization Sequences (NLSs) in the C-terminal domain of APC. A study in mice with mutation in nuclear localization signals of APC resulted in increased rates of cellular proliferation and increased expression of Wnt target genes [137].

In addition to the role of APC as a scaffold in the Destruction Complex, APC was found to sequester β-catenin in the nucleus, blocking its interaction with TCF thereby preventing the transcription of Wnt target genes such as *cyclin D1* and *c-myc*, which are involved in cell cycle regulation, cell proliferation etc [138, 139]. APC with its nuclear export signals acts as a chaperon and export β-catenin from the nucleus preventing its nuclear

localization and thereby its interaction with TCF [95, 140, 141]. APC may downregulate Wnt/β-catenin signaling by cytoplasmic retention of β-catenin [142].

APC was found to be associated with C-terminal binding protein (CtBP) through its conserved 15aa repeats. APC/CtBP complex diverts β-catenin away from TCF thereby inhibiting TCF-mediated transcription [101]. APC associate with a repressor complex and facilitate CtBP-mediated repression of Wnt target genes [143]. An examination of the effects of APC mutations on β-catenin phosphorylation, ubiquitination, and degradation in the colon cancer cell lines which contain different APC truncations,concluded that APC regulate β-catenin phosphorylation and ubiquitination by distinct domains and by separate molecular mechanisms [144]. In 2002, it was revealed that amino acids 1338-1555 of APC is involved in the differential regulation of the dephosphorylation and degradation of phosphorylated β-catenin[145].

Several other roles had been proposed for the role of APC in the Destruction Complex. Protein phosphatase 2A (PP2A) dephosphorylates β-catenin restricting its binding with SCF(β-TrCP) for ubiquitination. It was suggested that APC protects the N-terminal phosphorylated serine/threonine residues of β-catenin from PP2A and promotes β-catenin ubiquitination [146]. Mendoza-Topaz et al. [147] suggested that APC plays an important role in restricting the interaction of Axin with Dishevelled thereby preventing Dishevelled-mediated Axin recruitment to the plasma membrane. APC was found to compete with LEF-1 for β-catenin binding in the nucleus [103, 143, 148, 149]. Another model suggested that APC enhances β-catenin destruction by promoting Axin multimerization [150]. Liu et al. [120] stated that Siah-1 through its interaction with the C-terminal of APC promotes degradation of β-catenin, independent of the Destruction Complex.

Increased doses of β-catenin by specific APC mutations resulted in loss of ability and sensitivity of Embryonic Stem (ES) cells to differentiate [151]. APC mutations were found to differentially affect the capacity of stem cells to differentiate in a dosage-dependent fashion [152]. Wnt/β-catenin signaling pathway activation through loss of APC resulted in increased proliferation and defective differentiation of neural stem cells (NSCs) [153]. Differentiation was found to be blocked in hematopoietic stem cell on constitutive activation of Wnt/β-catenin signaling pathway [154]. Aberrantly

proliferating cells were found to reacquire self-renewal and multi-lineage differentiation capability on APC restoration in established tumors [155].

1.4.3.2 APC in cell adhesion

In 1993, APC was found to be associated with both β-catenin and α-catenin. As both α-catenin and β-catenin bind to E-cadherin, the cell adhesion molecule, it was suggested that APC is involved in cell adhesion [122, 123]. In 1995, it was suggested that a fraction of the APC protein is localized in the lateral cytoplasm of intestinal epithelial cells and functions in co-operation with catenins, whereas the APC protein in microvilli and in the apical cytoplasm has other functions independent of catenins. These findings gave an important link between the role played by APC in tumor initiation and cellular adhesion [156].

Hulsken et al. [157] demonstrated that E-cadherin and APC directly compete for binding to β-catenin. The interaction of E-cadherin and APC complexes to the cytoskeleton was found to be mediated by the N-terminal domain of β-catenin by binding to α-catenin. Plakoglobin or γ-catenin was also found to mediate identical interactions. Jou et al. [158] reported that the cell-cell adhesion molecule E-cadherin's cytoplasmic domain shares the SLSSL sequence which is found in four of the seven 20aa repeats of APC.

Presence of APC in the apical and immediately subapical regions and also along the lateral margins of certain cells, supports the role of APC in signaling at the adherens junction and indicates that APC plays a role in cells committed to terminal differentiation [159]. Drosophila APC homologue, E-APC was identified which was found to be concentrated in apicolateral adhesive zones of epithelial cells along with Armadillo and E-cadherin. E-APC requires actin filaments for its association with junctional membranes. The association of E-APC with adherence junctions was found to be necessary for E-APC in destabilising Armadillo [160, 161].

Efstathiou et al. [162] suggested that Trefoil factor 3 (TFF3), also known as intestinal trefoil factor which is a member of the trefoil family of peptide, may modulate epithelial cell adhesion through its interaction with APC and E-cadherin in complexes. It was reported that on overexpression of ASEF, ASEF complexes with truncated APC and down regulate E-cadherin-mediated cell-cell adhesion [163].

Studies by Carothers et al. [164] on Apc(Min/+) mice enterocytes which expressed a truncated APC allele showed reduced association between E-cadherin and β-catenin and decreased levels of E-cadherin at the cell membrane supporting the role of APC in regulating adherens junction structure and function in the intestine.

Stable expression of wild type APC in SW480 colon cancer cells that normally express a truncated form of APC (SW480APC) by Faux et al. [165] resulted in the translocation of E-cadherin to the cell membrane suggesting APC's role in cell adhesion by regulation of the intracellular transport of junctional proteins. Their studies suggested that APC might be involved in post-translational regulation of E-cadherin localization. Their results also pointed another interesting role of APC - the inhibition of cell growth. β-catenin was found to translocate from the nucleus and cytoplasm to the cell periphery, and reduced β-catenin/TCF mediated transcription and inhibition of cell growth was observed. SW480APC cells were found to reach a stationary phase where the proliferation is greatly reduced or ceased entirely at a lower cell density, which is suggestive of contact inhibition of growth [166]. Contact inhibition is a key anticancer mechanism that arrests cell cycle or proliferation when cells reach a high density. Contact inhibition refers to two distinct but closely related phenomena: Contact Inhibition of Locomotion (CIL) and Contact Inhibition of Proliferation (CIP). When two cells comes in contact with each other, they attempt to move away from each other in a different direction to avoid future collision (CIL). When collision is unavoidable, growth of the cells eventually stops in a cell-density dependent manner (CIP). Duration of cell cycle and cell size is affected by many signaling pathways including the Wnt/β-catenin signaling pathway in which APC plays key regulatory role [167].

Bienz et al. [168] suggested that, APC as a β-catenin binding protein may have a role in the choice of β-catenin between cell adhesion and Wnt/β-catenin signaling and may have separate functions in Wnt/β-catenin signaling and cell adhesion. APC was found to regulate the distribution of β-catenin between Wnt/β-catenin signaling and cell adhesion pools through its ability to shuttle between nucleus, cytoplasm and membrane [169].Thus loss of cell-cell adhesion may be another way by which loss of function of APC promotes carcinogenesis.

1.4.3.3 APC in cell migration

A cell's cytoskeleton is composed of actin filaments or microfilaments, microtubules and intermediate filaments. Coordinated regulation of these cytoskeletal elements is necessary for a cell to perform diverse cellular functions like cell adhesion, cell migration, and cell division. The actin filaments is thought to provide mechanical strength to the cell, microtubules form a polarized network allowing organelle as well as protein movement throughout the cell and intermediate filaments provide a supporting framework within the cell and are considered to be the most rigid component responsible for maintaining the overall shape of the cell. APC is found to be associated with all of these cytoskeletal elements providing evidence for its role in cell migration. The ability of cells to migrate is critical for maintaining tissue homeostasis in colonic crypts.

Smith et al. [170] revealed APC protein as a filamentous network which extended throughout the cytoplasm and co-localized with microtubules. They also suggested that wild-type APC protein may be associated with the microtubule cytoskeleton. Munemitsu et al. [171] demonstrated that only wild-type APC protein binds to and affects the assembly of microtubules, while APC mutants was found to lack this activity.

Immunofluorescence microscopic studies in 1997, revealed the localization of full-length APC protein in the cytoplasm and also in the nucleus of human mammary epithelial cells. The cytoplasmic APC protein was found to be concentrated at the leading edge of migrating cells [172]. APC was found to move along microtubules and assemble as granules at their growing plus ends [173]. Microtubule stability was found to increase on binding of APC to microtubules and was found to be regulated by phosphorylation of APC. GSK3β/CKI-phosphorylated APC was found to localize to microtubule-dependent clusters at the tips of membrane extensions and the loss of GSK3β/CKI-phosphorylated APC from these clusters was found to result in decreased cell migration [174, 175].

In 2002, Barth et al. [176] suggested that assembly of APC into cortical clusters may be mediated by the APC armadillo domain. Nathke et al. [177] showed that APC protein localizes mainly to clusters of puncta near the ends of microtubules that extend into actively migrating regions of epithelial cell membranes. APC protein containing

membranes were found to be actively involved in cell migration. In the intestine, at the crypt/villus boundary, where cell migration is crucial, APC protein levels were found to increase. A sharp decrease in migration along the crypt-villi axis was observed in enterocytes in the small intestine of APC (Min/+) mice [5].

APC was found to complex with the kinesin superfamily KIF3A-KIF3B, the microtubule plus-end-directed motor proteins, through the kinesin superfamily-associated protein 3 (KAP3). For accumulation of APC in clusters, its interaction with KAP3 was also found to be essential. These findings lead to the conclusion that KAP3-KIF3A-KIF3B complex transport APC and β-catenin along microtubules facilitating its accumulation at the tips of membrane protrusions, and thus may control cell migration. Loss of APC was found to be associated with decreased protrusion formation [91, 178].

In 1998, Morrison et al. [179] revealed the close association of end-binding protein 1(EB1) with the microtubule cytoskeleton throughout the cell cycle. APC was found to bind with EB1 and the interaction between APC and EB1 targets APC to microtubule tips [180, 181]. Microtubule polymerization is an important factor in the growth and stability of microtubules. EB1 was found to promote microtubule polymerization in permeabilized cells in the presence of C-terminal EB1 binding domain of APC and hence the interaction between EB1 and APC was suggested to play crucial role in the regulation of microtubule polymerization [182]. APC protein was found to bind non-assembled tubulin with high affinity and induces the tubulin assembly and the bundling of the assembled microtubules [115]. Formin mDia which is an effector of Rho GTPase was suggested as a scaffold protein for EB1 and APC in stabilizing microtubules and in promoting cell migration and microtubule network polarization. APC membrane recruitment 2 (Amer2), and anterograde kinesin KIF17 are other co-factors implicated in APC-EB1 interaction and microtubule stabilization [183-185].

APC was found to accumulate at actin ruffles and contribute to actin organisation and polymerisation [186-188]. Actin filaments and microtubules were found to compete for binding to the C-terminal basic domain of APC, suggesting a possible role for APC in cytoskeletal cross-talk [116]. APC was found to directly nucleate actin assembly and thereby promote directed cell migration and microtubule-induced focal adhesion

turnover [189, 190]. APC was found to bind intermediate filaments and was reported as a vital regulator in organization of intermediate filaments during cell migration [191].

Phosphorylation of APC by Glycogen Synthase Kinase-3β(GSK3β) was found to decrease interaction of APC with microtubules [174]. Thus Wnt/β-catenin signaling may inhibit APC's role in cell migration. In 2005, Penman et al. [192] suggested that the interaction of APC with microtubules and the β-catenin-targeting complex are mutually exclusive, and indicate that the distribution of endogenous APC between different pools is dynamic, which allows cells to distribute it as required.

For a migrating cell, polarized organization of its cytoskeleton towards the direction of migration is vital. Cell polarity must be properly oriented for a cell to move through the crypt-villus axis in a given direction. In 2003, studies in migrating astrocytes, found that association of APC with microtubules is essential for cell polarization. Cdc42-dependent phosphorylation of GSK3β occurs specifically at the leading edge of migrating cells and induce APC protein to interact with microtubule plus ends. From these observations it was concluded that Cdc42 regulates cell polarity through the spatial regulation of GSK3β and APC. It was also suggested that this role may contribute to APC's tumour-suppressor activity [193]. One of the studies in APC-knockdown epithelial cell lines resulted in loss of polarity and multi-layering on permeable supports, and enlarged, filled spheroids with disrupted polarity in 3D culture and hence it was suggested that tumor initiation due to APC mutation may be due to loss of polarity and dysmorphogenesis [194].

Watanabe et al. [92] in 2004 suggested a model in which activation of Rac1 and Cdc42 in response to migration signals leads to recruitment of IQGAP1 (an effector of Rac1 and Cdc42) and APC which, together with CLIP-170 (a microtubule-stabilizing protein), form a complex that links the actin cytoskeleton and microtubule dynamics during cell polarization and directional migration. APC was found to localize to the basal cortex and the basal APC puncta was found to be aligned with cortical microtubules and were often seen at end-to-side junctions of microtubules. APC has been proposed as a component of a template that guides microtubule network formation in polarized epithelial cells and may also function to capture and direct the growth, organization and stabilization of microtubules at the cell membrane in epithelial cells [195]. Interaction between APC

and Dlg1, an ortholog of Drosophila melanogaster discs large protein was found to be essential for polarization of the microtubule cytoskeleton in migrating astrocytes [196]. APC was found to interact with microtubule-binding nucleoporin, Nup358 that regulates APC localization to the cell cortex and was found to act in polarized cell migration. APC was found to interact with Nup153 at the nuclear membrane and serves as an anchoring site for microtubule association with the nuclear membrane. Nup358 and Nup153 play critical roles in centrosome reorientation during migration [197, 198].

In 2000, Kawasaki et al. [90] found that the protein product of a cloned gene, ASEF (Rac1-exchange factor) directly interacts with the armadillo repeat domain of APC leading to cell flattening, membrane ruffling, and lamellipodia formation. It was suggested that APC-ASEF complex may regulate the actin cytoskeletal network, cell morphology and cell migration. Another study of Kawasaki et al. [163] reported that mutant APC was found to be more efficient than wild-type APC in stimulating the activity of ASEF which may trigger Rac-dependent cytoskeletal reorganization and loss of E-cadherin at cell-cell junctions, and thereby promoting cell migration. Recovery of E-cadherin at cell-cell junctions which can be correlated with increase in cell adhesion on restoration of wild-type APC expression in SW480 (APC 1-1337) CRC cells was reported by Faux et.al [165]. And hence these studies suggest that APC mediate tumorogenesis by gain of function too in addition to its loss of functions.

1.4.3.4 APC in the regulation of mitotic spindle assembly and orientation

EB1 that interacts with the APC tumor suppressor was found to be closely associated with the microtubule cytoskeleton throughout the cell cycle. EB1 was found to colocalize with cytoplasmic microtubules in interphase cells and to spindle microtubules during mitosis. Hence it was suggested that EB1 together with APC may play a role in the regulation of normal growth and differentiation processes in the colonic epithelium [179, 199]. EB1 was suggested to promote growth and interactions of microtubules within the central spindle and at the cell cortex [200]. APC and EB1 was found to localize to mother centriole [201].

The APC-related molecule Kar9, which was reported as a cortical protein required for cytoplasmic microtubule orientation in Yeast was found to be essential for proper alignment of the spindle with the mother-bud axis in budding yeast. Cells that lack or

with a mutant form of Kar9 was shown to have defects in spindle orientation [202, 203].Kar9 was also found to interact with EB1[204, 205]. Abnormal spindle behaviour was noticed on deletion of another microtubule-binding protein, Bim 1(the yeast homologue of EB1 which is a binding partner of APC) [206]. Kar9 was found to direct microtubule orientation by interacting with Bim1. In yeast, a cortical microtubule-capture site was identified with both Kar9 and Bim1[207-210].

For symmetric epithelial division along the planar axis in Drosophila epithelium, APC and EB1 were found to be essential [211]. It was suggested that during mitosis, APC modulate microtubule plus-end attachments. Even a single mutant APC allele may lead to highly aberrant mitotis [212]. In FAP crypts the population of mitotic cells were found to be extended upward into the crypt middle as compared to normal crypts which are found to be located in the bottom-third [213]. Truncated APC, APC(1-1450) was found to act as a dominant negative in the regulation of spindle dynamics and chromosome alignment. APC(1-1450) was demonstrated to form a hetero-oligomer with the full-length APC thereby preventing it from its association with EB1[214].

During studies in Drosophila preblastoderm embryos dAPC2 and actin condensations were observed at different stages of mitosis. These condensations above the plane of the spindle helps in their interaction with the astral microtubules. Drosophila homologs of APC, dAPC2 and dAPC1 were found to be expressed in dividing cells of the larval brain. In larval neuroblasts, both dAPC2 and Arm were found to colocalize to a cortical crescent next to the future daughter cell. dAPC2 was also found to be asymmetrically localized in embryonic neuroblasts.dAPC1 was found to localize to centrosomes and microtubules. These findings implicated cytoskeletal roles for these drosophila homologs of APC, dAPC1 and dAPC2 [215, 216] .

APC was found to localize to the centromere and the kinetochore region of mitotic chromosomes[217]. Studies in mouse Embryonic Stem (ES) cells demonstrated APC accumulation at the kinetochore during mitosis. APC mutant cells were found to form mitotic spindles with a large number of microtubules that cannot efficiently connect with kinetochores [218].

Cortically localized APC in Drosophila germline stem cells helps in asymmetric stem cell division by playing significant roles in regulating the orientation of mitotic spindles

perpendicular to the stem cell niche [15]. Cortical APR-1, the APC homolog in Caenorhabditis elegans was found to reduce force generation by stabilizing microtubule plus-ends at the cell cortex and also acts as an attenuator of the pulling forces acting on the mitotic spindle during asymmetric cell division [219].

It was demonstrated that APC interacts with the microtubule-nucleation factor γ-tubulin and also stimulate and stabilize microtubule growth using its C-terminal sequences [220]. The rate of APC recycling at the centrosome was found to be enhanced by microtubule growth and hence may be a positive feedback to stimulate its role in microtubule growth [221].

Cyclin A/cdk2 was found to interact with APC in late G2 phase of the cell cycle and phosphorylates APC at residue Ser-1360 in the Mutation Cluster Region (MCR) and this phosphorylation is found to influence the astral microtubule attachment to the cortical surface in mitosis [222]. APC inactivation was found to result in abnormal mitosis and up-regulation of BUB1B and MAD2L1, 2 genes involved in the mitotic checkpoint. APC was found to contribute to normal compaction of mitotic chromatin in meiotic Xenopus egg extract and HeLa cells [223, 224].

It was demonstrated that APC mutations in human colorectal cancers prevents mitotic spindle anchoring at the anaphase cortex and inhibit cytokinesis by blocking initiation of the cytokinetic furrow [225]. In budding yeast with mutated motor protein Dynein, the mitotic spindle is often found to be improperly positioned leading to delayed cytokinesis. YEB1, the Yeast homologue of the human APC binding protein EB1, was suggested to contribute to the sensor mechanism that activates a checkpoint to eliminate this delay in cytokinesis [226]. It was shown that in the beginning of anaphase, APC binding protein, EB1 together with Dynactin, the protein that mediates the microtubule capture events promote elongation of astral microtubules and also contribute to the stimulation of the cleavage furrow in cytokinesis in dividing sea urchin eggs [227]. Even though the exact mechanisms by which how APC regulates mitotic spindle assembly and orientation remains unclear, these many findings provide evidence for APC as a key player in the regulation of mitotic spindle assembly as well as its orientation.

1.4.3.5 APC in chromosome segregation and chromosomal instability (CIN)

Allelic imbalance of chromosome 5q21 and others were noticed during studies in early colorectal tumors and hence chromosomal instability was suggested to exist very early during colorectal tumorigenesis and the role of APC in chromosomal instability(CIN) was speculated [228]. Studies in APC mutant mouse embryonic stem cells revealed increased chromosomal abnormalities. It was suggested that loss of sequences that lie C-terminal to the β-catenin regulatory domain in APC may lead to chromosomal instability in CRC [218]. During mitosis, APC was found to play regulatory roles in kinetochore-microtubule attachment by forming a complex with the checkpoint proteins, BUB1 and BUB3 at kinetochores. APC was revealed as a high-affinity substrate for BUB Kinases. Truncated mutations in APC gene resulted in aberrant segregation of chromosomes [229]. In APC mutant CIN cells of the colon, spindle damage was observed suggesting that APC may contribute to chromosomal instability in colon cancer [230]. Even a single mutant APC allele may lead to defective microtubule plus-end attachments that leads to highly aberrant mitotis and chromosomal instability [94]. Changes in tubulin distribution in spindles and asters and reduced microtubule density was observed in APC depleted Xenopus extracts suggesting significant roles for APC in microtubule binding, spindle formation and chromosome segregation which when defective may lead to chromosomal instability [231]. APC inhibited cells were found to compromise the mitotic spindle checkpoint leading to reduced interaction of BUB1 and BUBR1 with kinetochores resulting in tetraploidy and polyploidy [232].

From the insights gained while studying six kinetochore-bound MT-associated proteins (kMAPs) including APC and EB1, Draviam et al. [233] proposed that defective EB1 or APC may lead to aneuploidy. APC mutant cells were shown to have only reduced capability to correct errors in the kinetochore-microtubule attachments [234]. Studies in N-terminal APC fragments (N-APC) HCT-116 colon cancer near diploid cell line, with two wild-type APC alleles resulted in premature exit in mitosis, spindle checkpoint defect, spindle damage and aneuploidy that can lead to chromosomal instability. It was suggested that the initial APC mutation may destabilize the genome and also may set the stage for deregulated proliferation upon loss of the second APC allele [235]. In Xenopus egg extracts N-APC mutant was found to interact with MAD2, an essential mitotic checkpoint protein and inactivates mitotic checkpoint. Expression of N-APC mutant in

mammalian cells with an intact mitotic checkpoint resulted in premature anaphase onset with missegregated chromosomes [236].

1.4.3.6 APC in the regulation of cell cycle

Overexpression of APC was found to block cell cycle progression from G0/G1 to the S phase of the cell cycle and this was associated with overexpression of cyclin E/CDK2 or cyclin D1/CDK4 kinases. It was suggested that APC may negatively modulate the activity of cyclin-CDK complexes and play critical roles in the regulation of the cell cycle [237]. It was suggested that transient overexpression of full length APC induces G1 phase arrest in colorectal cancer cells and this was found to be associated with a loss of β-catenin–induced transcription. APC-induced changes in gene expression of Wnt target genes or proliferative genes such as *cyclin D1* and *c-myc* may be sufficient to cause G1 arrest. Disruption in the control of G1/S progression by APC mutation is found in most colon tumors [238]. APC was also proposed to affect proliferation in a β-catenin–independent manner. And hence it was suggested that mutant APC that fails in maintaining G1/S checkpoint control may contribute to aberrant cell proliferation.

APC was found to interact with Topoisomerase IIα (Topo IIα), which is a critical regulator of G2/M transition through the 15aa repeat region (M2-APC) and also 20aa repeat region (M3-APC). Cell cycle arrest was noticed in cells expressing M2-APC in the G2 phase of the cell cycle. Expression of M3-APC resulted in G2 cell cycle accumulation. Purified recombinant M2- and M3-APC could considerably enhance the activity of Topo IIα. Expression of either of the amino acid repeats M2- or M3-APC also led to increased aneuploidy in cells with full-length endogenous APC [239, 240]. Mutant APC lacking the S/TXV motif, with which APC binds to the PDZ domain of human homolog of the Drosophila discs large tumor suppressor protein (hDLG), was found to have less effect on cell cycle blocking than full length APC. Overexpression of hDLG was found to suppress cell proliferation by blocking cell cycle progression from the G0/G1 to S phase. It was suggested that APC-hDLG complex may negatively regulate cell cycle progression from the G0/G1 to S phase [241].

1.4.3.7APC in the regulation of DNA repair and apoptosis

Base Excision Repair (BER) is the DNA repair evolutionarily conserved pathway that facilitates the repair of damaged DNA via two general pathways: “single nucleotide

BER," also referred to as "Short Patch (SP)-BER," and "multinucleotide (2–13-nucleotide repair patch) BER," also referred to as "Long Patch (LP)-BER. The Short Patch BER pathway leads to the repair of a single nucleotide whereas the Long Patch BER pathway leads to the repair of at least two nucleotides. APC was found to modulate the BER pathway through its interaction with DNA polymerase β (Pol β) and flap endonuclease 1 (Fen-1) and mediate CRC cell apoptosis. APC may determine whether cells with DNA damage survive or undergo apoptosis. A proliferating cell nuclear antigen-interacting protein-like box sequence in APC was identified that binds DNA polymerase β and blocks DNA polymerase β-mediated strand-displacement synthesis in LP-BER without affecting SP-BER. Hence it was suggested that APC distinguishes between the LP-BER and SP-BER pathways and plays critical roles in determining its utilization and suggest a mechanism by which the LP-BER and SP-BER pathways may be regulated differentially within the cells. APC was found to interact with Pol-β through amino acid residues, ILe1259 and Tyr1262 in the N-terminal region of APC and this domain is known as the DNA Repair Inhibitory (DRI) domain. It was suggested that colonic tumors or cell lines harbor truncated APC without DRI domain. It was also shown that the wild-type APC gene expression was required for DNA methylation-induced sensitivity of colon cancer cells. DNA methylating agents are potent mutagens and carcinogens. Increased APC protein levels was found to increase sensitivity of mouse embryonic fibroblast cell lines and human colon cancer cell lines to DNA methylating agent, methylmethane sulfonate resulting in blocked BER and increased apoptosis. Thus APC plays critical roles in DNA-alkylation damage-induced colorectal carcinogenesis [114, 242-246]. Increased assembly of BER proteins, as well as higher activity of Fen1 and Pol β was seen in LoVo, colon cancer cells expressing truncated APC protein [247]. APC was found to interact with replication protein A 32kDa subunit (RPA32) and modulate replication stress responses [248]. APC was also found to be directly involved in DNA Double Stranded Break (DSB) repair and truncation mutations was found to impair chromatin-associated functions of APC [249].

Cell death is equally important as cell birth so as to maintain tissue homeostasis in tissues like colonic epithelium which when disrupted may lead to cancer. When new cells are produced older cells gradually move to the upper crypt and are finally shed from the crypt lumen. Studies in normal colonic mucosa revealed that a considerably

high level of APC was found where matured cells reside [250]. A considerable decrease in cell growth due to induced cell death through apoptosis was noticed on expression of APC in human colorectal cancer cells with endogenous inactive APC alleles [251]. Studies in human colon cancer tissues and in human colon cancer cell lines revealed that apoptotic activity of Clusterin, which is a widely expressed glycoprotein that has both pro- and antiapoptotic activities was found to be regulated by APC. In colon cancer tissues without wild type APC, Clusterin RNA and protein levels were found to be low but when apoptosis was induced by wild type APC expression in these tissues, an increase in the Clusterin RNA and protein levels localizing to apoptotic nuclei was noticed [252]. In normal colonic epithelium, wild type APC was found to downregulate the expression of the antiapoptotic protein Survivin, which was found to be preferentially expressed in the lower crypt, through APC/β-catenin/TCF-4 signaling. It was thus hypothesized that "wild type APC, by progressively decreasing Survivin and increasing apoptosis from crypt bottom to top, may limit the population size of stem cells and other proliferative cells in the lower crypt; mutant APC may allow expansion of these populations, thereby initiating tumorigenesis [253]. Resistance to apoptotic-inducing agents was observed in human colon cancer specimens with mutant APC because of a reduction in the levels of Caspase-3, Caspase-7, and Caspase-9 which are proteolytic enzymes that trigger apoptosis. And apoptotic activity was found to be restored on restoration of these Caspase levels. It was hypothesized that one of the functions of APC is the regulation of Caspase activity and other apoptotic proteins by controlling their expression levels in the cell [254]. Caspase-8 was noticed as a vital component in APC-mediated apoptosis and also it was demonstrated that APC mediates apoptosis independent of β-catenin mediated transcription [255]. Chen et al [256] suggested that APC may induce apoptosis through the inhibition of β-catenin/TCF–mediated transcription. Inactivation of D-APC in Drosophila was found to inhibit retinal neuronal degeneration and apoptosis and this could be rescued by expression of Armadillo [257]. Valproic acid (VPA) - induced apoptosis in colon cancer cells, which is an inhibitor of histone deacetylases (HDAC) was found to be determined by APC [258]. Recent studies in HT29 colorectal cancer cells revealed that APC-dependent apoptosis requires the production of reactive oxygen species (ROS) by the mitochondrial respiratory chain [259]. Reduced apoptosis was observed on inhibition of APC in U2OS cells (human bone

osteosarcoma epithelial cells). APC inhibition was found to induce chromosomal instability after a combination of mitotic and apoptotic defects[232].

The induction of apoptosis caused by cell detachment with the extracellular matrix or upon cell adhesion to inappropriate location, termed as anoikis is a means of protecting the organism from improper cell growth. Anoikis resistance is thought to be critical for tumor progression. β1 integrins are transmembrane heterodimeric receptors that are mainly responsible for the establishment of extracellular matrix-cytoskeleton linkage and cell adhesion-mediated activation of signaling pathways. The disruption or loss of integrin-mediated cell adhesion induces "anoikis" [260]. APC was found to be associated with β1 integrins [261]. Anoikis was observed in colonic crypts and it was suggested that its dysregulation may play a significant role in CRC progression and crypt homeostasis [262]. Induction of anoikis was observed on blockage of E-cadherin binding [263, 264], while anoikis resistance was observed on overexpression of β-catenin in epithelial cells [166]. But the role of APC in anoikis remains undiscovered.

1.4.4 APC mutations in colon cancer

1.4.4.1 APC mutations in FAP

Small deletions accounts for 46% of the disease causing germline APC mutations, nonsense mutations accounts for 28% and small insertions accounts for 10%. In addition 3% missense mutations and 13% gross alterations (exon deletions and duplications) were also reported [265]. Majority of the germline mutations in FAP take place in the 5′ half of the APC gene resulting in truncated protein product mostly due to frameshift or nonsense mutation. The truncated protein products are expressed and are usually stable. The mutational hotspots in the APC gene are located at codons 653, 1061 and 1309 in the 5′ part of exon 15 [266, 267].

FAP tumors follow the Knudsen two-hit hypothesis. FAP patients are found to inherit one germline mutation and develop tumors from those cells whose second APC allele is lost (second hit) following somatic mutations or Loss of Heterozygosity (LOH) [268, 269]. LOH was reported as a common genetic event in many types of tumors [270, 271]. LOH at the APC locus is a frequent event in both sporadic and FAP and was reported in 30-40% of CRCs [272, 273]. LOH with copy number losses (CNL-LOH) occur by deletion of the wild type allele leaving a single copy of the mutated allele, whereas, LOH with

copy number neutral (CNN-LOH) occur by loss of the wild type allele and duplication of the mutant allele, resulting in two copies of the mutant allele[274].

Analysis of APC mutations in FAP patients has shown that the site of the germline mutation in APC, can determine the type and position of the somatic mutation or the 'second hit' in colorectal polyps [269]. LOH is the most likely second hit in FAP patients with germline mutations around codon 1300 particularly FAP patients with germline mutations at codon 1309 exhibit severe colonic polyposis [275]. The second hit was more likely an APC truncating mutation for FAP patients with germline mutations between codons 1250 and 1450 in the Mutation Cluster Region (MCR) of APC [269]. Hence bi-allelic APC mutations, leads to loss of protein function. Mutations that occur close to codon 1300, result in stable truncated proteins and provide strongest selective advantage to the cell in which they occur. These stable, truncated APC proteins lack all of the SAMP repeats involved in Axin/Conductin binding, disrupting β-catenin degradation, and only one of the 20aa β-catenin-binding/degradation repeats was found to be retained [269, 276]. In FAP patients when germline APC mutation resulted in truncated proteins without any of the seven 20aa repeats involved in down-regulation of β-catenin, the majority of the corresponding somatic point mutations was found to retain one or, less frequently, two of the same 20aa repeats. On the contrary, when germline mutation results in a truncated protein retaining one 20aa repeat, most of the corresponding second hits remove all 20aa repeats. There may be selection for bi-allelic hits to APC, with retention of 20aa repeats in at least one allele [277, 278]. Rosin-Arbesfeld et al. [141] reported an inverse correlation between the level of TCF/β-catenin transcriptional output and the number of 20aa β-catenin repeats in APC in CRC cell lines, which support the ability of truncated APC proteins to down-regulate Wnt/β-catenin signaling with less effect. Hence from all these findings, it could be concluded that in FAP adenomas a specific level of Wnt/β-catenin signaling is produced that is optimal to drive carcinogenesis through selection for truncated APC protein that are likely to retain some activity in down-regulating β-catenin signaling [277, 279, 280].

1.4.4.2 APC mutations in sporadic CRC

Around 95% of all CRC cases are sporadic CRCs. Most of the understanding regarding FAP colorectal cancers is applicable to sporadic CRCs too but there are major

differences too. Somatic mutations in the APC gene is reported in around 70% of sporadic CRCs [44, 280, 281]. Around 90-95% of APC mutations detected in sporadic CRC's are truncating, with nonsense and frameshift mutations and at times splice site mutations [280-282]. Missense or silent APC mutations accounts for around 10% of sporadic CRC cases [280]. LOH at APC is found in approximately 35% of sporadic CRC cases [281]. Around 50-60% of APC mutations in sporadic cancers occur in the Mutation Cluster Region (MCR) [44, 273, 280]. Interdependence of hits was also reported in sporadic CRC cases with evidence for one hit that left two intact 20aa repeats, and the other hit that removed all the 20aa repeats [278].

1.5 The Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway is an ancient, evolutionarily and highly conserved pathway involved in both vertebrate and invertebrate embryogenesis, cell-to-cell signaling and also in the regulation of adult tissue homeostasis [283]. Wnt/β-catenin plays critical roles in the regulation of cell fate determination, cell proliferation, cell differentiation, cell migration, cell apoptosis, cell polarity etc. Even though aberrant Wnt signaling is reported in many types of cancers, the role of Wnt/β-catenin signaling in tumorigenesis is most notably detailed for colorectal cancer and is described as a key regulator of both early and later stages of colorectal cancer progression. The name Wnt is a fusion of the name of the gene that was found to control segment polarity during larval development in Drosophila, the wingless gene (wg) [284] and the name of the vertebrate homolog, Wnt1 (originally named Int-1) [285].

Wnt/β-catenin signaling pathway or the Canonical Wnt Signaling Pathway is considered to be the most important Wnt Signaling pathway. Wnt/β-catenin signaling pathway regulates gene transcription through the cofactor β-catenin, which is the key transducer of the Wnt signal from the cytoplasm to the nucleus and enhances the expression of oncogenes, like *c-Myc*, *cyclin D1* etc which are essential for cancer progression [286, 287]. Wnt/β-catenin signaling pathway is the primary event in colon cancer progression and around 90% of colon cancer cases result from aberrant Wnt/β-catenin signaling and high levels of β-catenin accumulation resulting from mutations in the APC gene [288].

There are 19 Wnt genes in human genome that encode a family of secreted glycoproteins with sequence homology having around 350–400 amino acids each with conserved glycosylation sites and also with a conserved pattern of 23-24 cysteine residues [289]. Wnt proteins can act in short range as well as in long range signaling by forming a concentration gradient and different concentrations of Wnt proteins elicit distinct cellular responses. Wnts undergo lipid modifications that helps in their interaction with receptors [290].

The transcriptional coactivator, β-catenin is the central and essential component of the Wnt/β-catenin signaling pathway [291]. The pathway is concentrated on the maintenance of the level of cytoplasmic β-catenin through its phosphorylation by a mutiprotein Destruction Complex thereby targeting it for degradation by proteasome and its regulation by Wnt ligands. The mutiprotein Destruction Complex consists of Adenomatous Polyposis Coli (APC), Casein Kinase 1 (CK1), and Glycogen Synthase Kinase 3β (GSK3β) and the scaffolding protein Axin that brings together all these components to close proximity[107]. Axin is present in low levels in the cytoplasm indicating Axin as the rate limiting factor for the formation of the Destruction Complex [292]. The CK1 and GSK3β phosphorylate Axin, APC and also β-catenin to target it for degradation. APC binds to β-catenin through its 15aa and 20aa repeats [102, 104, 122, 123, 131, 293]. Phosphorylated and nonphosphorylated APC can bind β-catenin. The phosphorylation of the 20aa repeats of APC by GSK3β and CK1 enhances its affinity for β-catenin by 300- to 500-fold [103]. SAMP repeats in APC mediate Axin binding [106, 107]. The binding of Axin to APC enhances the phosphorylation of APC by GSK3β and CK1 [293]. APC mutations within the region containing the 20aa repeats and just upstream of the SAMP repeats resulting in truncated proteins as seen in most of the colorectal cancer cases interrupt the activity of the Destruction Complex leading to constitutive activation of the Wnt/β-catenin signaling pathway.

The distribution of β-catenin between degradation, signaling and cell adhesion is regulated by phosphorylation. β-catenin is sequentially phosphorylated by CK1 and GSK3β in the Destruction Complex. β-catenin bound to the Destruction Complex gets phosphorylated at Ser45 by the priming kinase CK1 followed by GSK3β mediated phosphorylation at Tyr41 and consequent phosphorylation at residues Ser37 and Ser33 by GSK3β [294, 295]. Phosphorylation at Ser33 and Ser37 of β-catenin by GSK3β serves

as recognition sites for the β-TrCP, a subunit of the SCFβ-TrCP E3 ubiquitin ligase complex. The SCFβ-TrCP ubiquitin ligase poly-ubiquitinates β-catenin, leading to β-catenin degradation via the proteasome pathway [296, 297]. Once β-catenin gets degraded the Destruction Complex becomes free to degrade more β-catenins. It is reported that APC transfer phosphorylated β-catenin to the ubiquitin ligase and mediates the disassembly of the Destruction Complex. And this disassembly recycles the Destruction Complex to degrade more β-catenins [298]. Reports from structural studies demonstrated that rather than by complex disassembly Destruction Complex is recycled just by its structural transformation [299].

Protein Phosphatase 2A (PP2A) is another component of the Destruction Complex that associates with APC and Axin [89, 300] and is found to positively (by dephosphorylating β-catenin and APC) [146, 293] and negatively (by dephosphorylation of other Wnt pathway components) regulate Wnt/β-catenin signaling [89].

Upon Wnt stimulation, Wnt ligand binds to the N-terminal extracellular cysteine rich domain (CRD) of the seven-pass transmembrane domain cell-surface receptor Frizzled (FZD) [301, 302]. FDZ receptors also has an intracellular C-terminal PDZ domain. The human genome encodes ten FZD receptors [303]. Wnt ligands also binds to co-receptor single-pass transmembrane low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (LRP5/6 in vertebrates and its Drosophila ortholog is Arrow). Wnt induces Fzd-LRP5/6 heterodimerization. In addition to this Wnt ligands induce dual phosphorylation at LRP5/6 PPPSPxS motifs by GSK3-β and CK1-γ [304, 305]. These dually phosphorylated PPPSPxS motifs at LRP5/6 serve as docking sites for Axin and recruits Axin to the plasma membrane [306, 307]. Dishevelled (Dsh/Dvl) is a cytoplasmic scaffolding protein which has an Axin binding domain, DIX. FZD receptors recruit Dishevelled to the trimeric Wnt receptor complex which may facilitate the interaction of Axin with phosphorylated LRP5/6. It was suggested that as Dvl interacts with Axin, Dvl may be required for the recruitment of Axin to the plasma membrane [308]. It was reported that the interaction of FZD and Dvl are essential for Wnt-induced LRP6 phosphorylation [309]. It was also reported that FZD-Dvl interaction recruit Axin-GSK3 complex and initiates LRP6 phosphorylation by GSK3 [310].

Recruitment of Axin and/or GSK3 to the plasma membrane disrupts the formation of the Destruction Complex and hinders β-catenin degradation by which β-catenin accumulates in the cytoplasm and enters nucleus to trigger TCF mediated transcription. Recent experimental studies have demonstrated that under Wnt stimulation the Destruction Complex is recruited to the Wnt–receptor complex and remains active [311] but the β-catenin phosphorylation rates were reported to be very less [312]. APC and Axin helps in the cytoplasmic retention of β-catenin as well as export of β-catenin from the nucleus preventing transcription [142]. APC also play other critical roles in preventing TCF mediated transcription which is mentioned in Section 1.4.3.1. It was reported that phosphorylation of β-catenin at Y142 is needed for Bcl9 mediated nuclear translocation of β-catenin. Hence this phosphorylation may serve as a functional switch for β-catenins between cell adhesion and Wnt/β-catenin signaling [294, 313, 314].

Nuclear β-catenin acts as a co-activator for, the TCF/LEF (T cell factor/Lymphoid enhancer factor) family of transcription factors. TCF/LEF interacts with transcriptional co-repressors like Groucho and CtBP and repress Wnt targeted gene expression when β-catenin concentration is low [315]. But β-catenin accumulation and nuclear translocation promotes its binding with TCF/LEF displacing Groucho resulting in Wnt target gene expression.

Figure 1.4 gives a simplified view of the Wnt/β-catenin signaling pathway. Figure 1.4A shows that in the absence of Wnt signal, the Destruction Complex is formed in the cytoplasm that phosphorylates β-catenin, which is then ubiquitinated by β-TrCP and finally degradad by the proteasome. Figure 1.4B shows that, the formation of Destruction Complex is hindered upon Wnt stimulation resulting in accumulation and nuclear translocation of β-catenin that binds with transcription factor, TCF and initiate Wnt target gene expression.

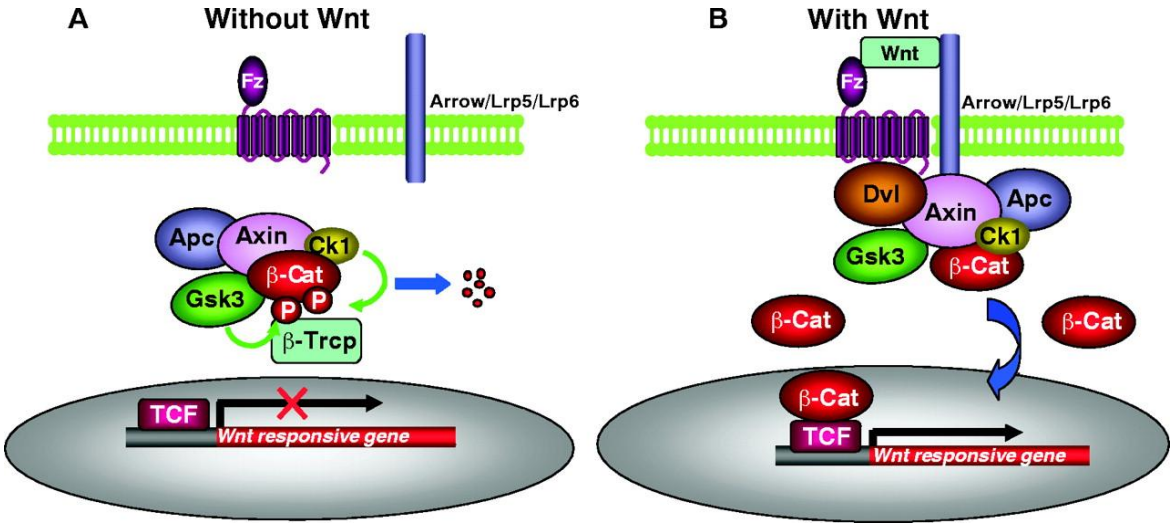


Figure 1.4 Overview of Wnt/β-catenin signaling pathway(Reproduced from Xi et al. (2004))[316].

1.6 Conclusion

In this chapter we have presented the biological background of our exploration of the multiple roles played by APC in the initiation of colon cancer. Even though a lot of biological information is available, the mechanisms that enable APC to maintain crypt homeostasis, as well as those that drive APC mutations to initiate colon cancer, remain unclear. Computational modeling provides a tool for evolving and testing different hypotheses on these mechanisms. In the upcoming chapters we present the computational models of colon crypt that we developed for simulating the multiple roles played by APC in maintaining normal tissue homeostasis and for exploring the role of APC mutations in the disruption of these processes and consequent initiation of colon cancer.

2

Computational Methods

This chapter gives a brief introduction to computational modeling as a powerful tool for research. The two computational techniques we use in this thesis, namely, Agent-Based Modeling (ABM) and Gillespie's Stochastic Simulation Algorithm (SSA) are described in some detail. A literature overview of existing models for the colon crypt is also presented.

2.1 Computational modeling and simulation in cancer research

Computational modeling refers to the use of computers to simulate and study the behavior of complex systems. Known features of the system and laws governing changes in the system are represented in the form of mathematical equations or rules. For creating a virtual simulation of the system, the governing rules or equations are written in the form of computer codes that enable the computer to solve the equations and yield the outcomes. The variables characterizing the model are given numerical values and the computer program is executed. The variables can be changed and the effect of these changes on the outcomes of the simulations can be observed just as in the case of real experiments. Computational models and simulations can therefore be used as virtual experimental facilities to perform experiments that are often very difficult or impossible in reality, for generating ideas and hypotheses that can then be tested in real experiments. Computational models are able to study a biological system at multiple levels, at the level of molecular interactions or cellular interactions, to see how these lower level interactions create or affect complex tissue or organ level phenomena.

Biological systems are organized into multiple levels from subatomic particles which form atoms that form molecules and macromolecules, such as DNA and proteins, eventually leading to cell formation. Organisms are built from these cells that form

tissues, organs and organ systems. Complexity in cancer as a biological system is a result of the bi-directional cross-talk between its heterogeneous system components within themselves and also with their heterogeneous environments. This cannot be easily understood by using wet-lab experiments alone. A huge amount of data is available at these multiple levels with the advancement in high-throughput technologies and also from experimental findings. Colon cancer research has reached a stage at which these data at multiple levels are to be integrated so as to unravel the complex mechanisms behind its initiation and progression. Computational modeling and simulation can handle multiple intracellular and extracellular factors acting at different scales of time and space and hence can be used to untangle the complexities of cancer [317].

Computational cancer models usually incorporate four spatial scales namely atomic, molecular, microscopic and macroscopic (Figure 2.1). Studies at the atomic level focus on the dynamic structural events within proteins, peptides and lipids with spatial lengths in the order of nanometers and nanosecond timescales [318]. Whereas molecular level studies focuses on cell signaling or other cellular processes to study the dynamic nature of a set of interacting proteins rather than individual proteins [319] with spatial lengths ranging from nanometers to micrometers and timescales from microseconds to seconds. In microscopic level, cellular level, multicellular level and tissue level interactions are modeled with spatial lengths from micrometers to millimeters and timescales from minutes to hours. Microscopic models study cancer cell-cell interactions and their interaction with their environment. Finally macroscopic level (organ level) models focus on the dynamics of the tumor morphology, shape, extent of vascularization, and invasion, under different environmental conditions [320] with spatial lengths from millimeters to centimeters and timescales from days to years or even decades.

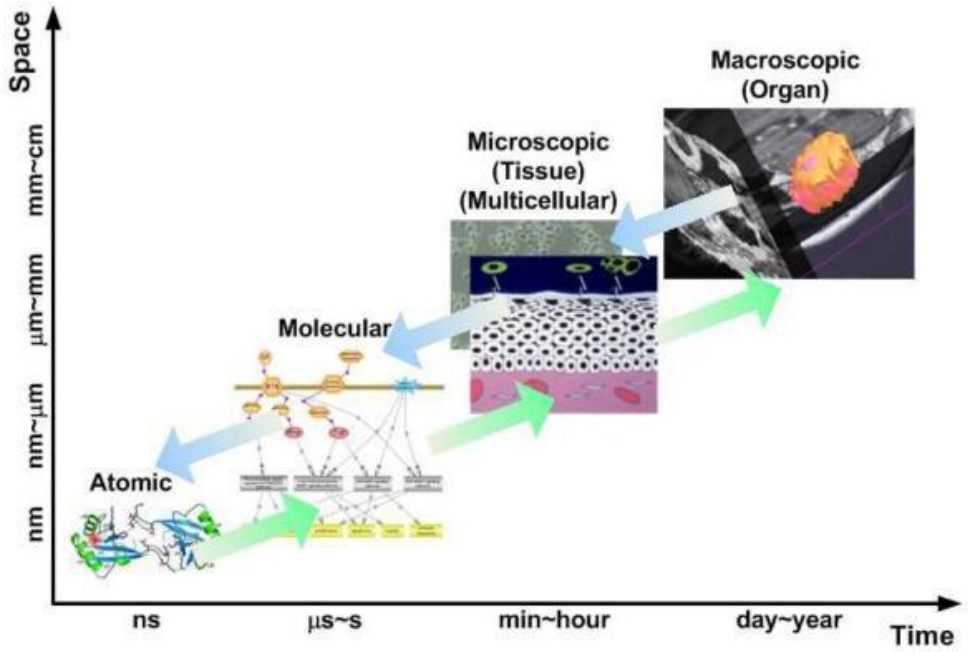


Figure 2.1 Pictorial representation of the hierarchical scales in cancer modeling. (Reproduced from Deisboeck et al. (2011))[321].

2.2 The different modeling approaches

Models can be either deterministic or stochastic. The final outcome of the deterministic model is fully determined by the parameter values chosen and the initial conditions, and therefore for the same set of parameters the model outcome will be the same for every execution of the model. The stochastic models exhibit randomness or uncertainty in behavior and hence the same set of parameter values and initial conditions can lead to a range of results. Complex systems like cancer are highly stochastic in nature and stochastic models are best opted in cancer modeling to account for the stochasticity.

Computational modeling of cancer involves modeling approaches that can be either discrete, continuum or hybrid, which is a combination of the discrete and continuum approaches. Discrete models operate at the scale of individual cells or cell clusters and represent components of a biological system as individual, distinct entities in space and time according to a specific set of biological rules. Discrete models are limited by the number of cells that can be handled and by the complexities associated. The continuum models operate at larger scales and describe cell populations rather than individual cells by means of continuous space at comparatively lesser computational cost. Hybrid models operate on a combination of both discrete and

continuum models. Computational models of cancer are recently built using hybrid approach incorporating both discrete and continuum approaches [322].

Traditional modeling includes graph-based techniques and equation-based techniques. Graph-based techniques are rarely used in modeling and include Petri nets and Pi-calculus [323, 324]. The modeling constructs of these techniques are quite primitive and hence cannot model complex systems efficiently. Graph-based techniques cannot handle spatial representation of the system with ease. They are also inefficient in representing priorities or ordering of events. Equation based modeling (EBM) include Ordinary Differential Equations (ODE's), Partial Differential Equations (PDE's) and Stochastic Differential Equations (SDE's). Equations become too complex to handle with increase in the complexity of the system. EBM's are not reliable if the system exhibits both discrete and continuous behavior. Also ODE's can efficiently model systems with large populations but they fail to imitate with perfection the complex interactions among smaller population of agents. ODE's are incapable of incorporating stochasticity and complexity of the system. Even though complex systems can be modeled using PDE's, the governing equations become too complex with increase in the complexity of the system and finding a solution becomes a huge challenge. SDE's, can efficiently model complex biological systems but are computationally very expensive. EBM's cannot model perfectly heterogeneous systems. Whereas Agent-based models (ABM's) can model systems that looks very similar to the system in reality.

2.3 Agent-based modeling and simulation

Agent-based modeling is a computational paradigm that can model individual components of a system and their interaction with each other and their environment. Agent-based models consist of interacting autonomous entities, or agents. Agents are defined by a set of attributes and rules that govern their behavior. Agents can interact with each other and with their environment (Figure 2.2) resulting in events leading to emergent collective behavior of the system. ABM's follow a bottom up approach as global behavior of the system is observed as a result of the individual agent actions [325]. The key building blocks of an ABM are the definition of the agent viz agent attributes and behavioral rules, the relationships between agents and the methods of their interactions, the agent environment and how the agents interact with the

environment [326]. During a simulation, an agent performs actions (event) that can change its own attributes, the attributes of other agents and also the environment parameters. Rules define how agents choose an action, update attribute values, and interact with each other and the environment.

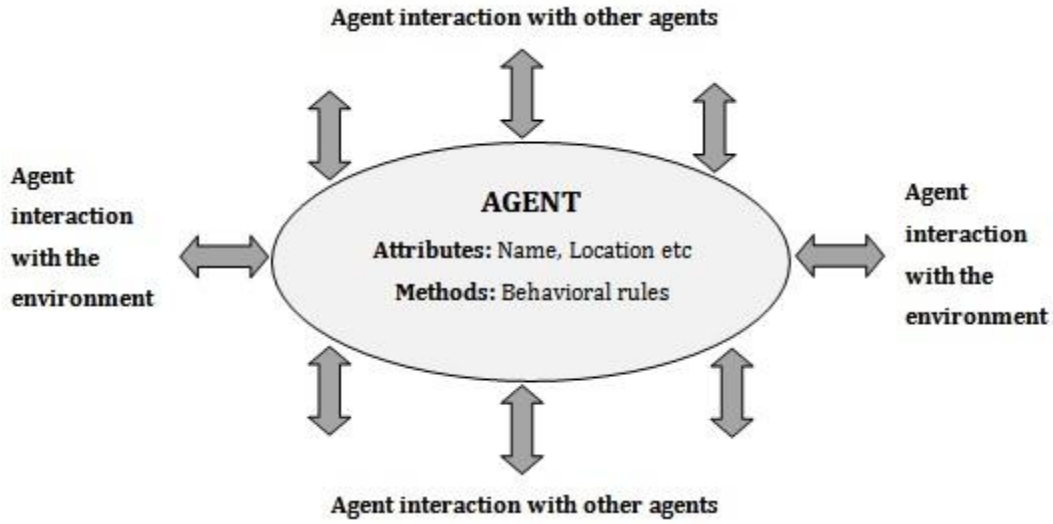


Figure 2.2 Schematic of the typical agent world

Before building an ABM, the modeler has to build a conceptual model by decomposing the problem into small manageable parts (decomposition), select the details of the problem to be modeled (abstraction), identify and manage the relationships among the components of the system (organization). After building the model, it is to be executed and then it should be verified, calibrated and validated (Figure 2.3). The model is executed repeatedly at specific time steps with same or different set of parameters and the consequence of every action is reflected in the simulation at every iteration (usually referred to as the time that represents one pass by the computer through the set of operations). An iteration can have different durations and may represent a second, a day or a year. Depending on the duration of the iteration, different behaviors can be implemented [327].

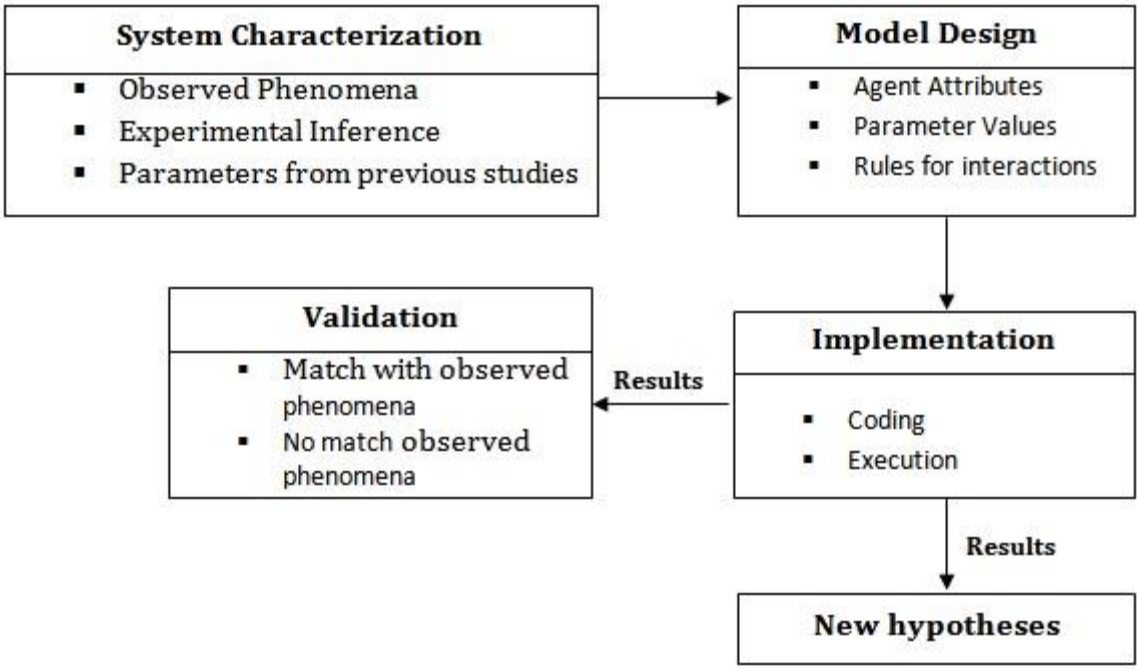


Figure 2.3 Flow diagram of the key steps in ABM development

ABM's, apart from capturing the emergent behavior of the system, can incorporate randomness and are flexible enough to represent the complex nature of the agents. Any criteria relating to an agent can be removed, added or modified at any time. ABM's can deal with single agents and groups of agents simultaneously with ease. ABM's can handle complex, nonlinear, discontinuous or discrete models. The interactions in ABM's can be changed dynamically. ABM's can model positive and negative feedbacks. With these advantages over traditional techniques, ABM's are well suited to model complex systems [328, 329]. Agent-based models can hence very well replicate the colon crypt to investigate the behavior of its individual cells as well as the crypt behavior as a system.

2.4 Features of agents

The characteristic features of agents are [326] :

Autonomous: Agents are autonomous and self-directed in their goals.

Modular: Agents are uniquely identifiable discrete individuals that are modular or self-contained having a boundary and decision-making capability.

Social: Agents interacts with other agents according to interaction protocols and its dynamic interactions with other agents influence its behavior.

Conditional: Agents have a state defined by their set of attributes and behaviors. State of the agents vary over time. Collective states of all the agents in the agent-based model along with the state of the environment constitute the state of the ABM. Agent behavior is conditioned on its state.

Goal-directed: Agents tries to achieve goals that drive its behavior.

Adaptive: Agents adapt its behavior based on previous experiences (learning and memory).

Heterogeneous: Agents have different properties, goals, experiences etc.

2.5 Gillespie's Stochastic Simulation Algorithm (SSA)

Traditional modeling approaches like ODE's which were used to simulate the time evolution of chemically reacting systems often fails to account for the inherent stochasticity in biochemical systems in living cells where small population of a few critical reactant species can result in discrete and stochastic behavior. Daniel Gillespie in 1976, introduced the stochastic simulation algorithm(SSA) [330, 331] , a popular computational method for stochastic simulations in biology which can numerically simulate continuous time Markov chains that models the dynamics of a system with low species count and uncertainty in transitions. SSA is an exact solution to generate the stochastic time evolution of a set of chemical species chemically reacting in a well-stirred environment as a random-walk process.

SSA can be described by considering a system in thermal equilibrium with N chemical species or reactants of the system (S_1, S_2, …, S_N) that are randomly distributed in a fix well-stirred volume (V) (reaction volume in which all the molecular species are homogeneously distributed and spatially indistinguishable) where the species interact through M chemical reactions (R_1,..., R_M). $X_i(t)$ denote the number of molecules of

species S_i in the system at time t. With SSA we can estimate the state vector $X(t)=(X_1(t),..., X_N(t))$, given that the system was in state $X(t_0)= x_0$ at some initial time t_0.

As a consequence of every reaction, the species population changes. Each reaction channel R_j is characterized by the state change vector, ν_j of the reaction and the propensity function a_j of the reaction.

State-change vector $\nu_j \equiv (\nu_{1j},...,\nu_{Nj})$, where ν_{ij} is the change in the molecular population of reactant S_i as a consequence of reaction R_j. A system in state x after reaction R_j immediately jumps to state $x + \nu_j$.

Propensity function $a_j(x)$ dt is defined to be the probability that one reaction R_j will occur in the next infinitesimal time interval (t, t + dt), given X(t)=x

The probability of randomly chosen molecules S_1 and S_2 that will react through a reaction R_j in the next infinitesimal time interval (t, t+dt) is c_j.dt, where c_j is the stochastic reaction rate constant (probability per unit time that S_1 and S_2 react).

Gillespie derived a combinatorial function h_j that specifies number of distinct combinations of R_j reactant molecules. The probability that an R_j reaction will occur in the interval (t, t+dt), is $h_j.c_j.dt=a_j.dt$, given that the system is in the state(X_1, …, X_N) at time t.

The time to the next possible reaction (τ) is an exponential random variable with mean (and standard deviation) $1/a_0(x)$, and index of the next possible reaction(j) is a statistically independent integer random variable with point probabilities $a_j(x)/a_0(x)$, given that the system is currently in state x. In SSA for any number of equations, at each time point, two random numbers are generated, r_1 and r_2 such that r_1 predicts the waiting time to the next reaction from an appropriate exponential distribution and r_2 decides which of the possible reactions will occur.

Hence,

$\tau = 1/a_0(x) \ln(1/r_1)$

j = the smallest integer satisfying $\sum_{j'=1}^{j} a_{j'}(x) > r_2 a_0(x)$given that the system is currently in state x[332].

With this generating method, the stochastic simulation algorithm (SSA)constructs an exact numerical realization of the process $X(t)$ as follows:

0. Initialize the time $t = t_0$ and the system's state $x = x_0$.

1. With the system in state x at time t, evaluate all the $a_j(x)$ and their sum $a_0(x)$.

2. Generate values for τ and j

3. Effect the next reaction by replacing $t \leftarrow t + \tau$ and $x \leftarrow x + \nu_j$.

4. Record (x, t) as desired. Return to Step 1, or else end the simulation.

2.6 Models for colon crypt

Many individual cell-based and multiscale models were developed to understand and to explore the processes at the subcellular, cellular as well as tissue level of the intestinal epithelium. Numerous models were developed to study cell proliferation, cell migration, cell differentiation, cell death as well as accumulation of mutations in the cells of the colon crypt.

Compartment models that used ODE's, studied populations of different cell types, but couldn't account for spatial regulation of cell behavior and also failed to model individual cells [333]. Continuum models that used PDE's to represent the spatial distribution of the different cell compartments also failed to model cells as discrete entities [334].

ABM's consider cells as individual entities and follow their actions and changes individually. ABM can be lattice based or lattice free, depending on whether the crypt is represented as a grid and cells are allowed to move only between fixed grid points or can move freely. Lattice-free models allow continuous movement of cells in space and include vertex models and cell-center models [335].

Examples of agent-based models of the colon crypt include Bomen et al. model that studied abnormal stem cell proliferation caused by APC mutation[336], a calibrated computational model that simulated cell dynamics in normal human colonic crypts[337], an agent-based model of colon crypt that studied how cell organization in crypts is achieved[338], a two-dimensional individual based model developed to predict cellular dynamics in human colonic crypts [339], an agent-based model of anoikis in the colon crypt [340] etc. Stochastic models were also developed that studied dynamics of the colonic crypt [341]. Multiscale multicellular models were built linking phenomena at the subcellular, cellular, and tissue levels of organization to study the crypt dynamics [335]. The computational as well as mathematical models for intestinal crypt were

thoroughly discussed, by Van Leeuwen et al. [342, 343], De Matteis et al. [344] , Kershaw et al [345] and Fletcher et.al. [346]. Several other colon crypt models were also developed in the recent years (Table 2.1). A few other models have explicitly modeled the effect of APC mutations on colorectal tumors (Table 2.2).

Table 2.1 Recent colon crypt models.

A mathematical model that reported early differentiation in colonic crypts [347].
A model that used optimal control theory, which is a mathematical optimization method to explore how populations of long-lived stem cells and shorter-lived progenies are maintained in specific proportions during adult life [348].
Mathematical models that studied the in vivo temporal dynamics of human intestinal stem cells [2, 349].
A model built using a theoretical and computational framework that studied colonic crypt behavior[350].
A spatially arranged linear process model that studied proliferation kinetics on the rate of somatic evolution in the colon [351].
A 3D lattice-free computational model that analyzed how Wnt signaling and contact inhibition affect proliferation [352].
A compartment model that studied monoclonality and the effects of the fixation of mutant clones [353].
A 3D hybrid stochastic model that investigated the mechanisms that may be responsible for homeostasis of intestinal epithelial tissue[354].

Table 2.2 Colon crypt models that include APC mutation.

A mathematical framework developed for the sequence of genetic events from APC mutation [355].
A mathematical model that estimated the relative growth advantages conferred by different combinations of APC mutations [356].
A spatial stochastic model that studied the rate of somatic evolution in a normal crypt with focus on the production of two-hit mutants that inactivate APC [357].
A PDE model that investigated abnormal crypt dynamics due to mutations in the APC gene [358].
A kinetic model that reported a dynamic interplay between β-catenin, APC, and Axin in the human colonic crypt [359].
A mathematical model that simulated adhesion and migration in APC mutated colonic crypts [360].
A stochastic model that studied crypt dynamics including consequences of mutations in different cell types and an analysis of the conditions for CIN to precede APC inactivation [341].
A mathematical model that studied three different Scenarios possible for the first two hits in APC [361].
A spatially arranged stochastic process model that incorporated a simple mutation model of APC [351].
Mathematical models of colon crypt that explained how APC mutations induce stem cell overpopulation and initiate colon tumorigenesis [362].
An agent-based model that studied the role of heterozygous APC mutation in niche succession and initiation of colorectal cancer [363].
An APC:Wnt counter-current-like model that explained how APC mutations induce proliferative abnormalities in colon cancer [47].

2.7 Conclusion

Colon cancer is a complex system of interacting components at multiple scales of length and time. The overall behavior of the system emerges from the interactions between its components at the lower levels, forming more complex behaviors as a collective. The emergent behavior of such complex systems can be reproduced in computer simulations, when we are able to define the right conditions or rules for the lower level interaction between its components. The computer simulations therefore serve as powerful tools for experimenting with different hypotheses on how the system works at

the lower levels. In the following chapters we present the computational models that we have developed for studying the multiple roles of APC in the initiation of colon cancer and how we use them as virtual experimental apparatus for conducting simulation experiments to understand the underlying phenomena.

3

Agent Based Model of the Normal Colon Crypt and Study of Niche Succession

This chapter describes a kinetic model which is developed to simulate the dynamics of the normal human colonic crypt. The modeling technique used is Agent-based modeling (ABM). The model is shown to reproduce the experimentally observed features of colon crypt dynamics. It is then used to simulate the phenomenon of niche succession in the colon crypt. Our interest in the phenomenon of niche succession in the context of this thesis on APC and colon cancer is due to two reasons:

1. Niche succession involves different modes of stem cell division and abnormalities of stem cell division play a significant part in the initiation of colon cancer.
2. APC is known to have a role in controlling the modes of stem cell division.

The phenomenon of niche succession is shown to emerge at the tissue level when we add a finite probability for stem cells to divide symmetrically. In later chapters we would be adding features to this model to simulate abnormalities caused by APC mutations.

3.1 Biological background

The intestinal crypts of Lieberkühn are closely packed, test tube-shaped invaginations that cover the surface of the intestine. Crypts are lined with a monolayer of epithelial cells arranged in a proliferative hierarchy and they house stem cells that are responsible for the rapid and constant renewal of the intestinal surface. The stem cell compartment is positioned at the crypt base, and comprises of slow-cycling stem cells [364]. The stem cells divide continuously, producing semi differentiated transit cells that move out of the niche. These semi-differentiated transit cells represent precursors at different stages of commitment and have the ability to divide rapidly. Therefore this cell

population is known as “Transit Amplifying” cells. On dividing a limited number of times, they get progressively differentiated and finally undergo terminal differentiation forming the different types of fully functional cells of the intestinal epithelium like colonocytes, goblet cells, enteroendocrine cells and paneth cells. At the same time, as new cells are produced in the stem cell niche the entire population of semi- and terminally-differentiated cells migrates towards the luminal orifice. Once the cells reach the crypt collar, they lose contact with the underlying basement membrane, extrude, undergo apoptosis [7, 8] and are shed from the luminal surface into the intestinal cavity. Abnormalities that cause imbalances in these processes of proliferation, migration, differentiation and shedding, can lead to tumors and to cancer.

At the molecular level, the Wnt/β-catenin signaling pathway that is triggered by Wnt ligands and regulated by APC, is considered to play the major role in cell proliferation in the crypt. It has been observed that the concentration of Wnt signaling molecules is maximum at the stem cell niche and reduces as we go up the crypt [152]. On the other hand, the APC expression within the cell increases as the cells get more and more differentiated. Both these factors work together in controlling the reduction of mitotic activity as we go up the crypt.

Niche succession is a phenomenon by which a crypt gets periodically overtaken by descendants from one stem cell. Analysis of the variability of methylation patterns that arise in a crypt during aging provide evidence for niche succession [17, 365]. Unlike the clonal succession associated with tumor progression which occurs due to selection of a particular lineage that carries proliferative mutations, there is no selection involved in niche succession; it is an entirely random process. However it is possible that it can give a piggy-back ride for tumor-initiating mutations and enable them to dominate the crypt well before tumor progression begins.

Two types of division are possible for stem cells: Asymmetric division and Symmetric division. In ‘‘asymmetric division’’, each stem cell generates exactly one stem cell and one semi differentiated (transit amplifying) cell at each division. The semi differentiated daughter cell leaves the niche to migrate up the crypt while the mother stem cell remains in the niche. In asymmetrical division, since the stem cells are always replaced, their lineages never become extinct.

Symmetric division, produces either two semi differentiated daughters that leave the niche or two stem cell daughters that remain in the niche. Stem cells have the ability to switch between asymmetric and symmetric modes of division. When the stem cell number in the niche goes down, symmetric division with two stem cell daughters that remain in the niche helps to bring the number up and when the stem cell number goes up, symmetric division with two differentiated daughter cells that leave the niche brings the number down. The balance between these two modes of division has been found to be defective in some disease states [366].

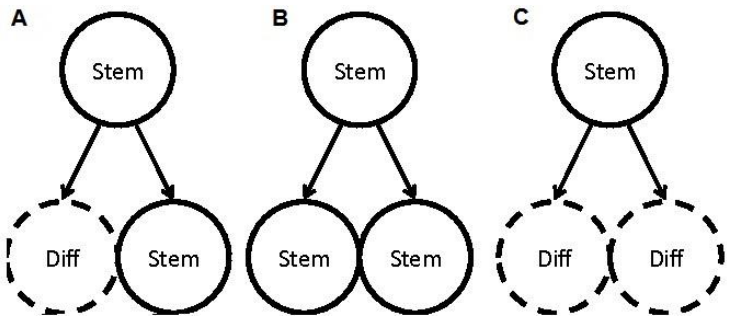


Figure 3.1 Asymmetric and symmetric stem cell divisions.(A) Asymmetric division producing one differentiated cell and one stem cell (B) Symmetric division producing two stem cells (C) Symmetric division producing two differentiated cells (Reproduced from Leili and Natalia, (2013)) [367].

Experimental studies have shown that APC has a role in the process of regulating the balance between asymmetric and symmetric cell division. APC plays this role through the influence it has on the mitotic spindle orientation [15, 368].

Niche succession is a consequence of symmetric stem cell division. Symmetric division can happen with two differentiated daughters or two stem cell daughters. With symmetric division, a stem cell lineage becomes extinct whenever both daughters are differentiated and leave the niche. To maintain a constant niche stem cell number, this extinction is balanced by expansion of another lineage by symmetric division in which both daughters remain as stem cells. This random stem cell loss with replacement can eventually lead to the extinction of all lineages except one, or ‘‘niche succession’’. The intra-crypt variability of methylation tags is indicative of the period of niche succession, greater variability showing slower niche succession [62].

3.2 The model

Traditional experimental methods are inadequate by themselves for studying the complex interactive nature of the processes within the crypt. Therefore a range of computational models have been developed to complement the experimental studies and these have been mentioned in Section 2.6 of Chapter 2.

Our model here is an Agent-based model (ABM) with the individual epithelial cells of the colon crypt as the agents. Simulations using the model are aimed at observing how assumptions of individual cell behavior affect the collective phenomena observable at the level of the crypt.

The normal crypt model is an adaption of the classical model developed by Potten and Loeffler in 1987 [369]. The crypt is represented as a simple 2D grid of dimension N x M which would be as if a cylindrical crypt is slit open and rolled out flat. The epithelial cells are anchored to the grid and can move on it. Each cell is an agent characterized by 7 attributes mentioned in Table 3.1.

Table 3.1 Agent attributes.

Attributes	Description
State	Specifies the cell-cycle stage and can take values as "Quiescent", "G1", "S+G2", "Mitosis".
Position	Specified by the (x,y) co-ordinates on the grid.
Time in State	Time spent in a particular state.
Age	Time that has passed since birth.
Number of Divisions	Number of times it has passed through mitosis.
Stemness	Defines the stage of differentiation/ determination of the cell.
Ancestor	The ancestral stem cell from which it has originated.

The model has eleven parameters namely,

1. Number of columns - N
2. Number of rows - M

3. Initial number of stem cells - N_0
4. Cell Cycle Time for Stem cells - TC_s,
5. Cell Cycle Time for Transit cells - TC_t,
6. Time in G1 state by Stem cells - $TG1_s$
7. Time in G1 state by Transit cells - $TG1_t$
8. Start of Wnt free zone - y_d
9. The maximum number of divisions before terminal differentiation - Num_div_max
10. Time step – Δt
11. Position of stem cell niche (y_0) and start of fully differentiated non-dividing cells (y_d).

The computer program that implements the model is developed on an ABM Framework developed in our group. The framework provides a platform for defining agents, their attributes, initial conditions and development of functions that govern the actions they perform. The rules for agent actions are thereby encoded in the functions. The framework is developed on VC.NET platform. The implementation is in VC++. The agents (crypt cells) are stored in the vector data structure. The information on the different types of cells, namely, stem cells, TA cells and differentiated cells are stored sequentially in the vector. The size of the vector is equal to the total number of agents (Figure 3.2)

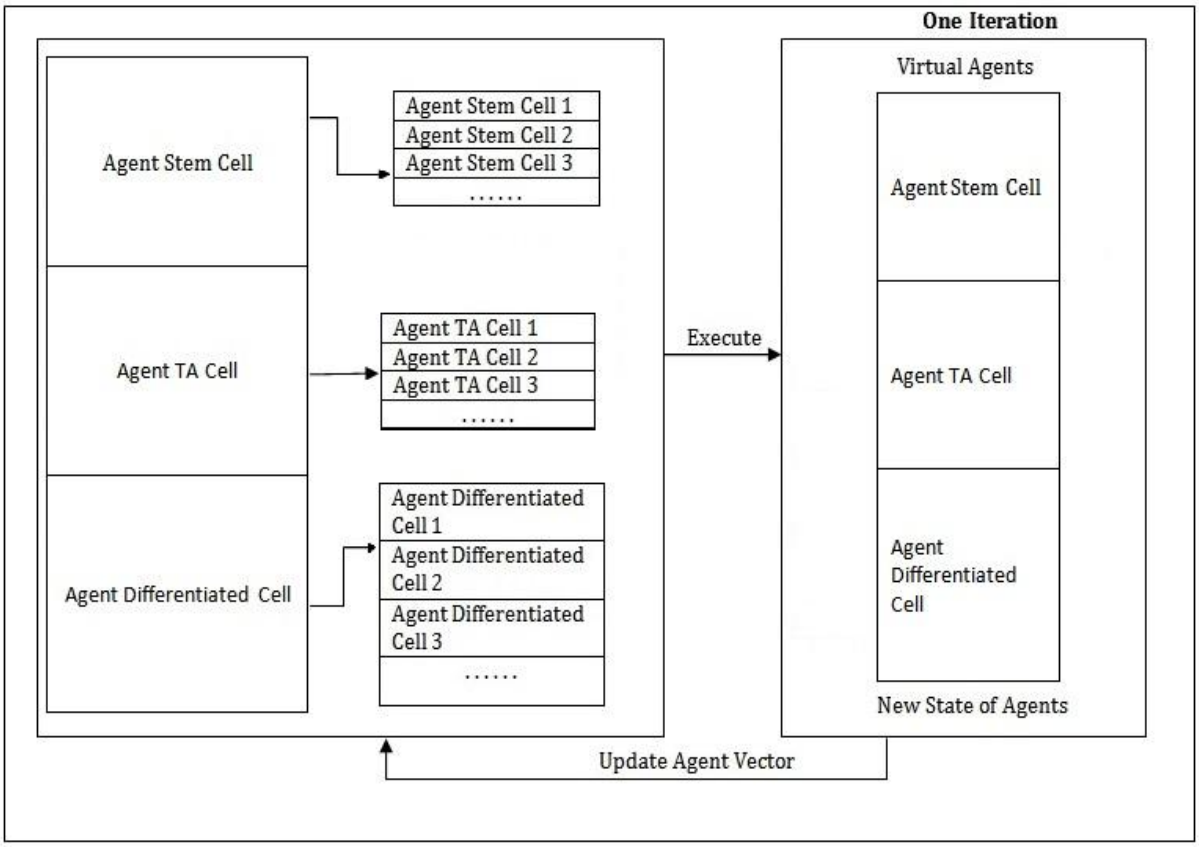


Figure 3.2 ABM implementation data structure.

A time loop is executed in which every agent and its environment (neighboring cells on the grid) is examined to see if the conditions for any action and consequent change of any attribute are fulfilled. The changes for all agents are effected together at the end of the time loop and the new time step starts with the changed conditions.

3.2.1 Implementing the role of Wnt signaling and APC in cell division.

The Wnt/β-catenin signaling pathway is assumed to control cell division and it is assumed that there is a gradient of Wnt signal across the height of the crypt with maximum signal value of 1 at the niche position y_0 and zero at the level y_d of the differentiated zone. The probability for a cell agent to shift from the "Quiescent" state and enter the cell cycle (P_{wnt}) is assumed to depend positively on the Wnt signal in its surroundings and negatively on the concentration of the APC regulatory protein expressed within the cell. The amount of APC protein expressed within a cell (APC_{conc}) is assumed to increase as "Stemness" value decreases.

We assume:

$APC_{conc} = 2 - Stemness$

Stem cells have a Stemness value of 1. Each cell division is assumed to reduce the Stemness of the progeny by 1/Num_div_max.

This gives APC_{conc} a minimum value of 1 for stem cells with Stemness=1 and a maximum value of 2 for fully differentiated cells with Stemness=0.

The input parameters used in the simulations are shown in Table 3.2. In order to save computation time only a portion of the colon crypt containing around 500 cells and a normal niche capacity of 10 stem cells is considered. Wnt signal concentration is assumed to become zero half way up the crypt axis.

Table 3.2 Parameter values.

Parameter	Value
Number of columns - N	10
Number of rows - M	50
Initial number of stem cells - N_0	10
Cell Cycle Time for Stem cells - TC_s	24 hours
Cell Cycle Time for Transit Amplifying cells - TC_t	12 hours
Time in G1 state by Stem cells $TG1_s$	12 hours
Time in G1 state by Transit Amplifying cells – $TG1_t$	4 hours
Start of Wnt free zone - y_d	50% of total height
The maximum number of divisions before terminal differentiation – Num_div_max	6
Time step Δt	30 minutes

3.2.1.1 Initial conditions.

The bottom row of the grid is considered as the stem cell niche. Initially N_0 stem cells are placed in the niche. All of them are initially assumed to be in G1 state but with different, randomly assigned values for the time that they have spent in that state. Therefore their division as well as the division of further generations is not synchronous. Time is incremented in steps of Δt.

3.2.1.2 Rules for division.

- If State is "Quiescent" State is changed to "G1" with a probability of:

  P_{wnt} = (1+Wnt)*(1-0.5*APC_{conc}), Where P_{wnt} is the probability of the transition from the quiescent state to the cell cycle, Wnt is the

concentration of Wnt signaling molecules in the environment of the cell and APC_{conc} is the concentration of APC expressed within the cell.

Time in State G1 is set to 0.

- If State is "G1" and Time in State<TG1, Time in State is incremented by Δt.
- If State is "G1" and Time in state >= TG1, State is changed to "S+G2" and Time in State is set to 0.
- If State is "S+G2" and Time in State< Cell Cycle Time – TG1, Time in State is incremented by Δt.
- If State is "S+G2" and Time in State >= Cell Cycle Time – TG1, State is changed to ‘‘Mitosis’’ and Time in State is set to 0.
- If State is "Mitosis", create and insert daughter cell and set State of both to ‘‘Quiescent’’

3.2.1.3 Rules for insertion of daughter cell.

It is possible for more than one cell to occupy a grid space; however daughter cells are preferably inserted into empty neighboring grid. If there is no empty neighbor, it is possible to insert it into an occupied neighbor also. The priorities for choosing the insertion position in the descending order are given below.

- Empty north neighbor
- Empty east or west neighbor (random choice between them if both are empty)
- Empty south neighbor
- Occupied north, east or west neighbor (random choice)

Stem cell daughters are inserted always only into east or west neighbor to ensure that stem cells do not leave the niche.

3.2.1.4 Rules for differentiation.

With each division the progenies get more differentiated and become less stem-cell-like. Therefore the ‘‘Stemness’’ value decreases from 1 to 0 as the stem cells divide to form semi-differentiated progenitor cells (transit amplifying cells) that divide several times before becoming terminally differentiated cells that cannot divide any more. The maximum number of divisions a progenitor cell can undergo before full differentiation is fixed by an input parameter ‘‘Num_div_max’’. The decrease in stemness per division is

given by 1/ Num_div_max. After terminal differentiation the cells remain in state ‘‘Quiescent’’.

3.2.1.5 Rules for migration.

The cells are assumed to move up by mitotic pressure. When new cells are born at the bottom, older cells get pushed up due to mitotic pressure. The model implements this through the rule that whenever more than one cell occupies the same grid space, the oldest cells are made to move up by one grid space. However stem cells never move out of the niche.

3.2.1.6 Rule for death/shedding.

Cells that reach the top row of the matrix are removed from the simulation to simulate cell shedding at the top of the crypt. Cells also die if they survive beyond a specified age.

3.2.2 Modeling symmetric stem cell division and niche succession

In the basic crypt model, stem cell division is considered to be purely asymmetric, each division resulting in one stem cell that remains in the niche and one differentiated cell which is capable of leaving the niche. We include the possibility of symmetric division as follows:

A parameter "symmetric division probability (P_s)" is defined as input. This parameter specifies the probability that a stem cell division is symmetric. Whenever a stem cell divides we generate a random number between 0 and 1. If the probability of symmetric division is greater than the random number the division is deemed symmetric. Symmetric division can be of two types with both daughters having Stemness = 1 or with both daughters equally differentiated. An additional parameter "differentiation probability (P_d)" decides whether the progeny will be two differentiated cells or two stem cells.

The probabilities that a cell division leads to 0, 1 or 2 stem cells are related to P_s and P_d as:

Probability that no stem cells are produced,

$$P_0=P_sP_d \tag{3.1}$$

Probability that one stem cell is produced (asymmetric division),

$$P_1=1-P_s \tag{3.2}$$

Probability that two stem cells are produced,

$$P_2=P_s(1-P_d) \quad (3.3)$$

For normal crypts the stem cell number needs to be maintained constant which is possible only if there are equal chances for both types of symmetric division. This implies that P_0 must be equal to P_2 and therefore the differentiation probability P_d should be 0.5. However in practice we found that setting the differentiation probability to 0.5 was not sufficient to maintain the stem cell number in our stochastic simulations. To ensure stability of the stochastic numerical computations we introduced a feedback factor to the differentiation probability that corrects for deviations of the actual stem cell number N_s from the original stem cell number N_0.

Differentiation probability,

$$P_d=0.5-(1-N_S/N_0) \quad (3.4)$$

The feedback factor $(1-N_s/N_0)$ ensures that when the number of stem cells goes above N_0 the differentiation probability increases above 0.5 producing more differentiated cells than stem cells and vice versa. P_d is limited to be >0.

Starting with N_0 stem cells, the time taken for all the stem cells to become descendants of one of the initial stem cells is taken as the niche succession period.

3.3 Results and discussion

3.3.1 Simulation results for crypt dynamics

Visualization of a crypt after it has reached dynamic equilibrium with birth rate being balanced by death rate and the total number of cells remaining steady in time, is shown in Figure 3.3. The number of cells in cell cycle diminishes as we move away from the stem cell niche.

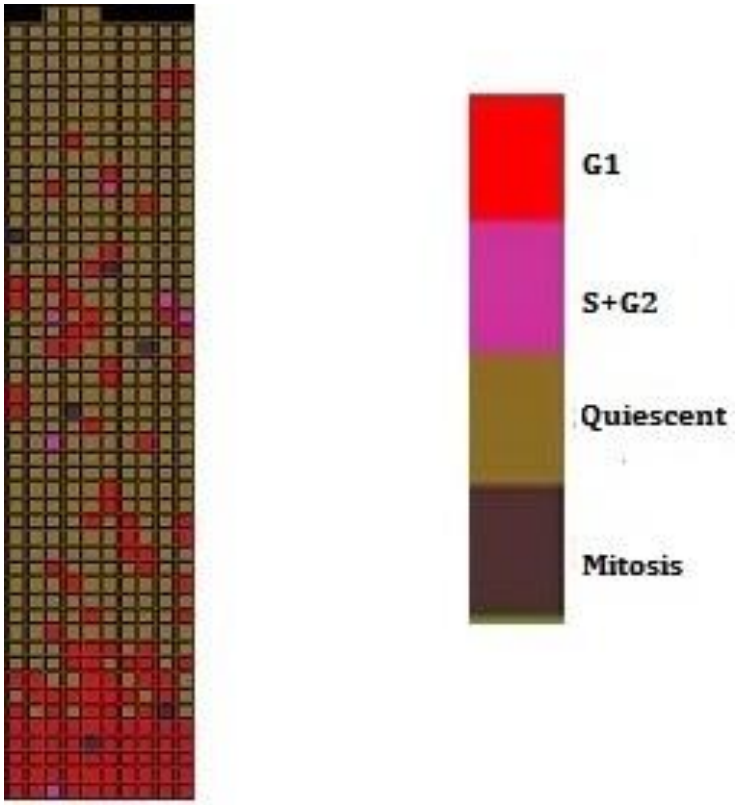


Figure 3.3 A simulated colon crypt under dynamic equilibrium. States of the cells are represented by different colors: Brown- "Quiescent", Red - "G1", Purple "S+G2", Dark Brown – "Mitosis", Black shows the gaps where there are no cells.

The relative distribution of quiescent and proliferating cells along the crypt axis is shown in Figure 3.4. It is clear that proliferating cells are limited to the lower region of the crypt thus reproducing real features of the crypt.

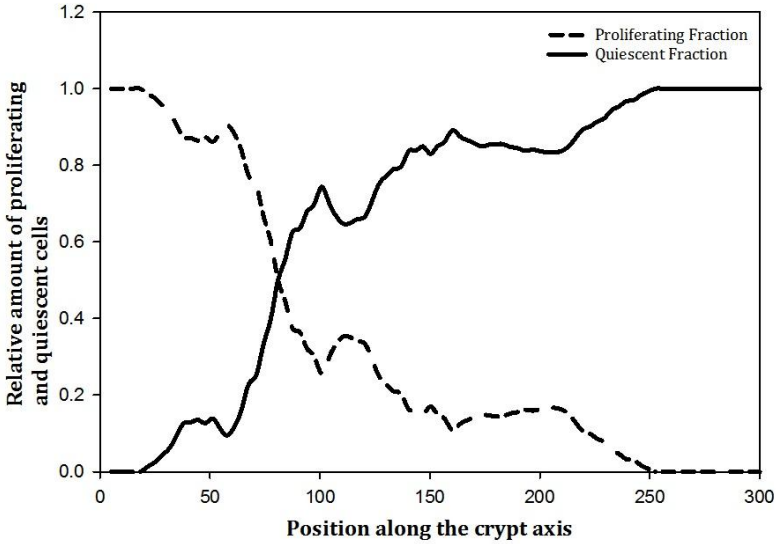


Figure 3.4 Relative distribution of proliferating and quiescent cells across the crypt axis. The proliferative hierarchy in the arrangement of cells is evident.

The total number of cells, the number of quiescent cells and number of cells in cell cycle are shown as a function of iteration steps in Figure 3.5. The numbers become steady in about 250 iterations. Under steady state conditions only about 25% of the total number of cells is in cell cycle at any point of time. This is an experimentally observed feature of the colon crypt[337].

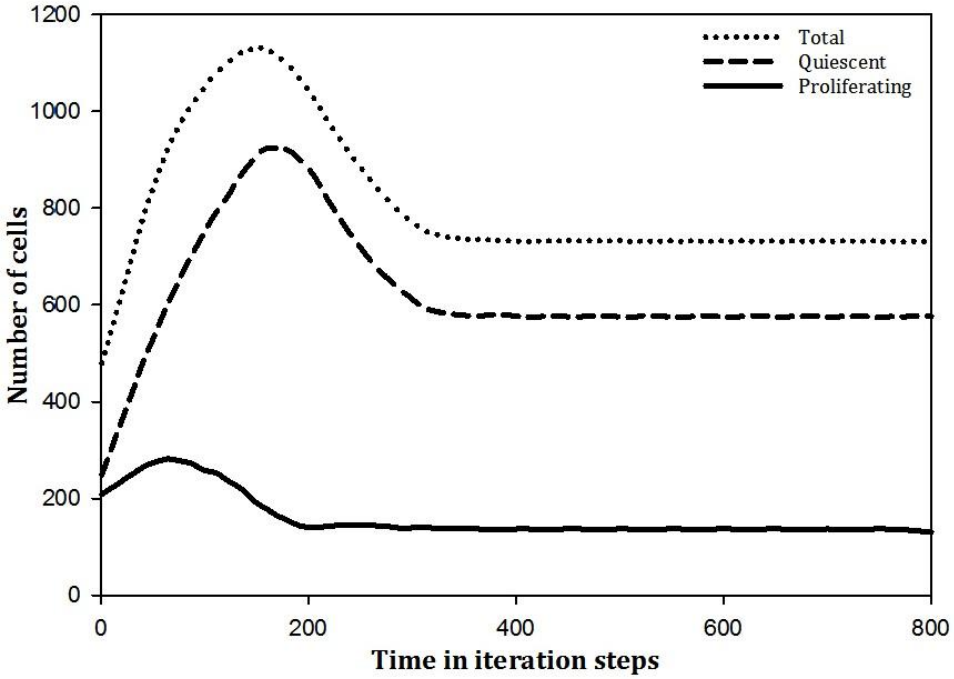


Figure 3.5 Cell numbers as a function of time (iteration steps)

The number of stem cells remains constant under the current assumption that stem cells divide strictly asymmetrically. In the next section the changes brought about by the introduction of symmetric division will be shown.

Figure 3.6 shows the migratory path of a cell from its birth to shedding. The cell is born into position y=75 μm at iteration number 12 and after waiting for about 32 iterations, it is pushed up by mitotic pressure from below and reaches the top of the crypt at iteration 93. The average velocity is about 7μm per hour.

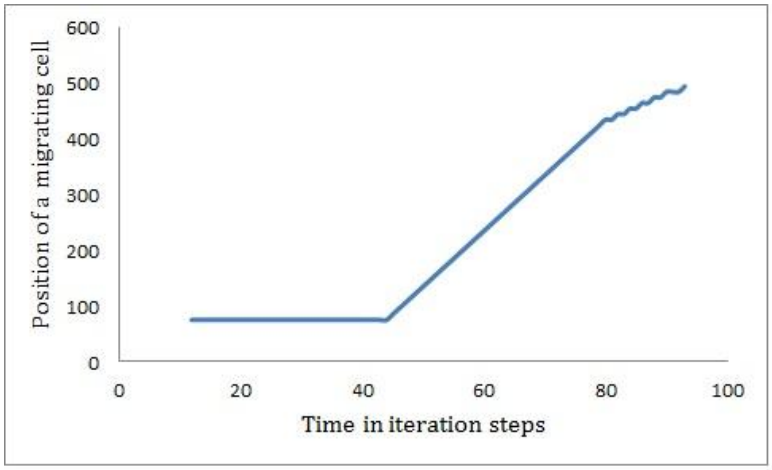


Figure 3.6 Trace of a migrating cell.

3.3.2 Results from simulations with symmetric division of stem cells

The simulation experiments determine how the niche succession period is affected by changes in the probability of symmetric division. To save computational time these simulations are done with a smaller system with N=10 and M=35.

We start with 10 stem cells of different lineages PC0 to PC9. The feedback differentiation probability (Eqn. (3.4) of Section 3.2.2) maintains the number of stem cells mostly in the 6–14 range with an average value of 10 (Figure 3.7).

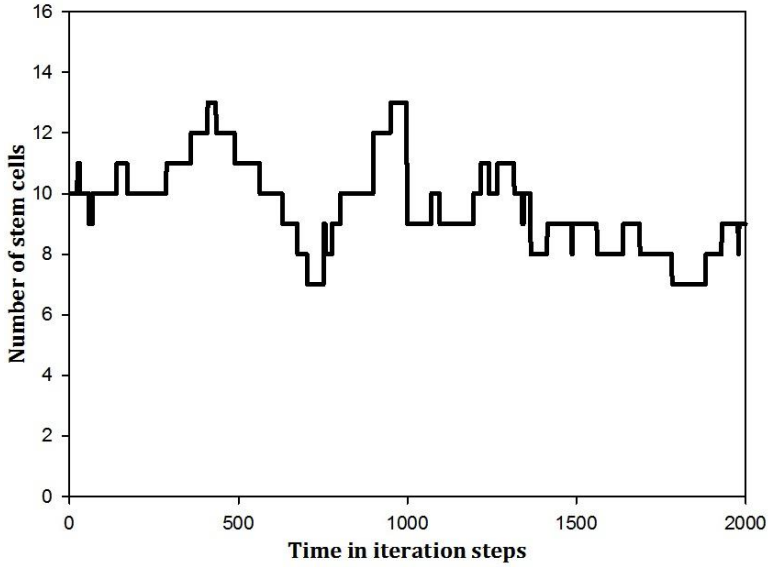
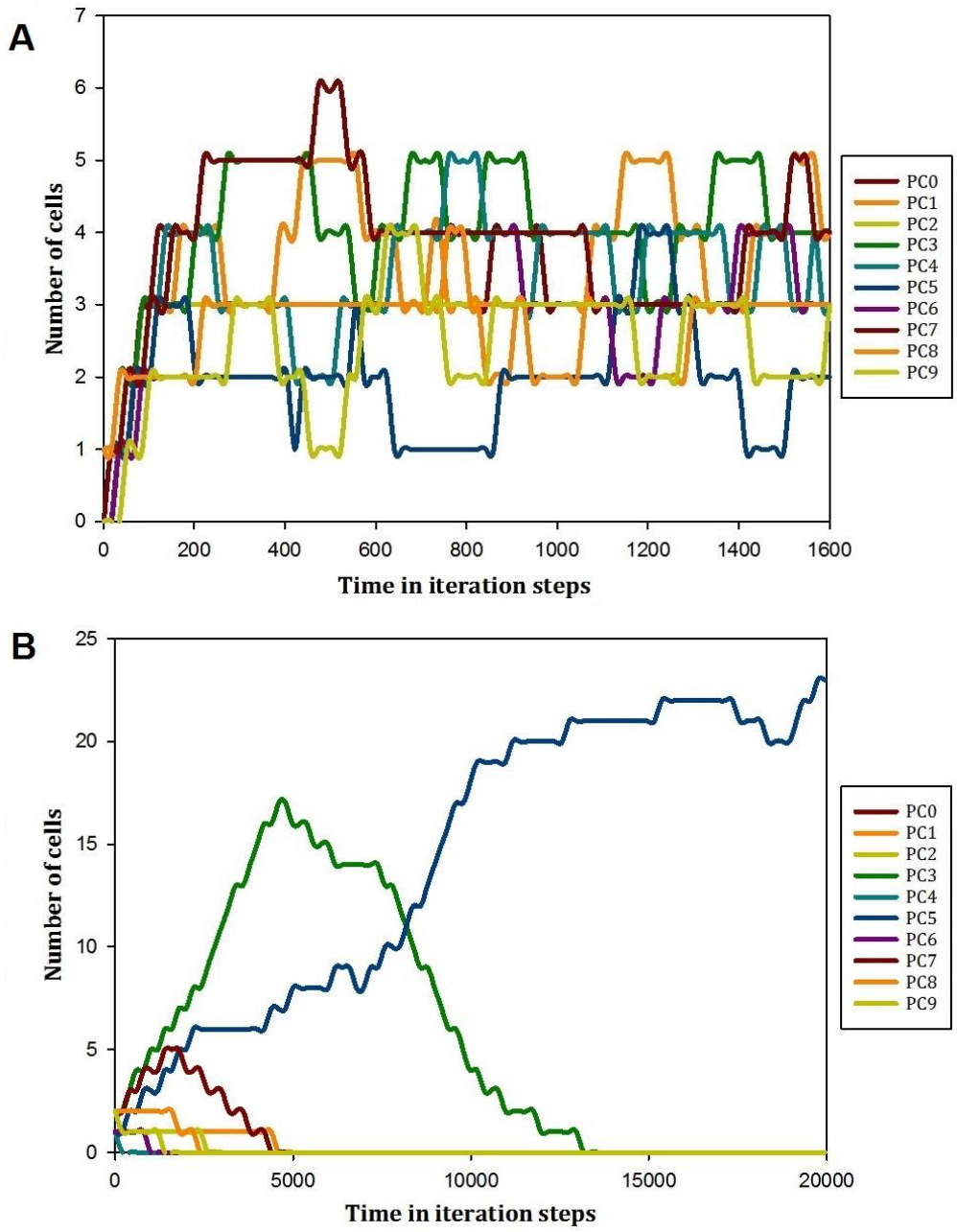


Figure 3.7 Stem cell number as a function of iteration steps.

The time taken for all the stem cells to become descendants of one of the initial stem cells is the niche succession period. Clonal succession happens when all

the non-stem cells in the crypt also becoming descendants of the dominant stem cell lineage that succeeds in capturing the niche.

Figures 3.8(A-D) shows lineage plots at four different values of the symmetric division probability. When there is no symmetric division (Figure 3.8A) there is no niche succession. As symmetric division probability increases, complete take over by a single lineage happens over shorter time periods i.e. the Niche succession period decreases (Figure 3.8(B-D)).



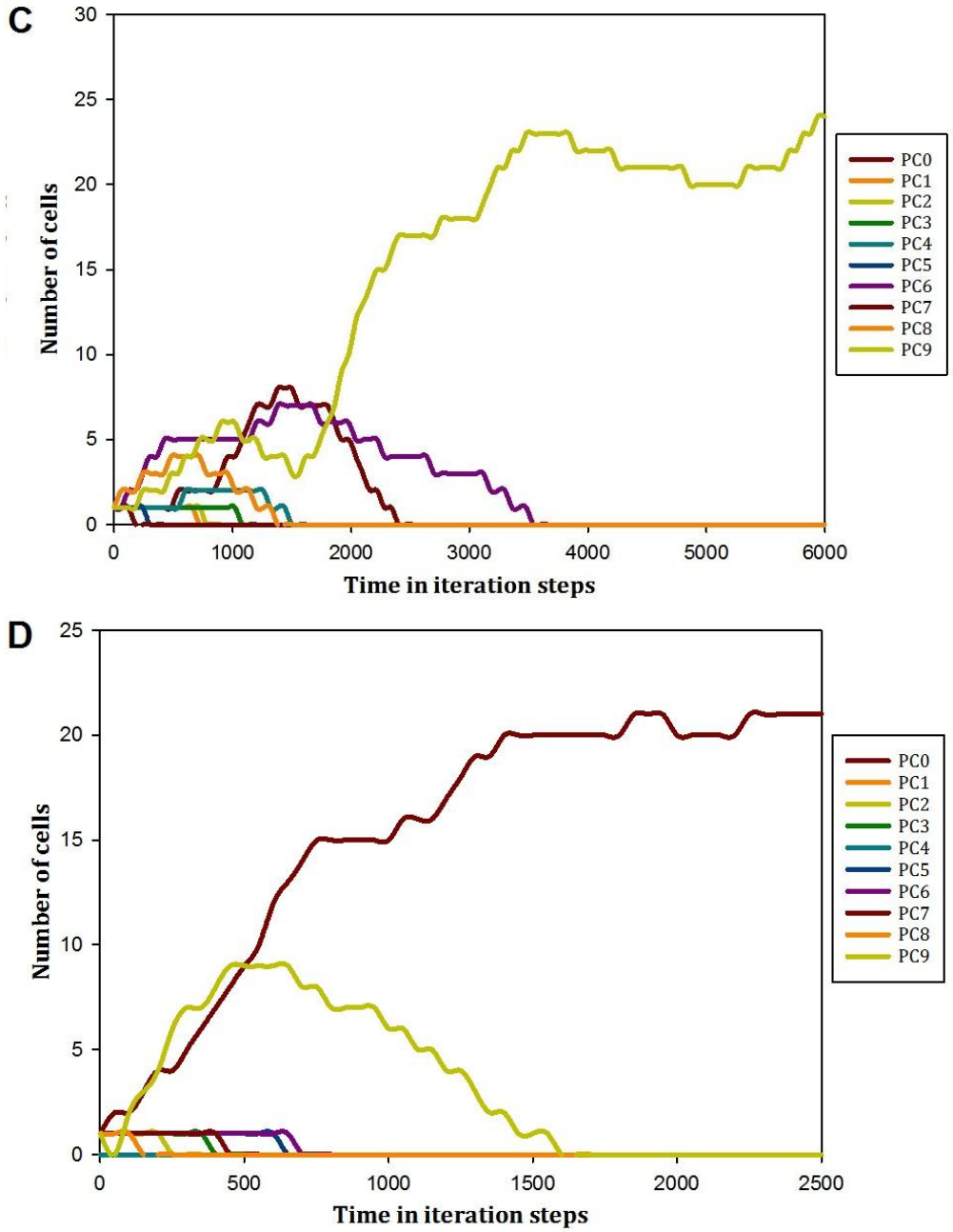


Figure 3.8 Lineage plots for different values of symmetric division probability (A) Symmetric division probability = 0.0 (B) Symmetric division probability = 0.1 (C) Symmetric division probability = 0.2 (D) Symmetric division probability = 0.4

Figure 3.9 shows variation plot of the average niche succession period with the symmetric division probability P_s (averaged over 10 simulations for each value of P_s). The niche succession period is seen to decrease steeply with increase of P_s for smaller values of P_s, but flattening as the symmetric division probability increases. For pure asymmetric division, (P_s = 0) there can be no extinction of

any stem cell lineage and therefore the niche succession period tends to infinity as P_s tends to zero. As P_s increases stem cell lineages acquire a finite probability for becoming extinct by symmetric differentiation and therefore the period of niche succession decreases. As P_s increases further the probability of survival of a lineage by symmetric production of stem cell progeny also increases along with the probability for extinction by symmetric differentiation. Therefore the slope of the curve decreases with increase of P_s. Similar trend of behavior has been obtained by van Leeuwen et al [342].

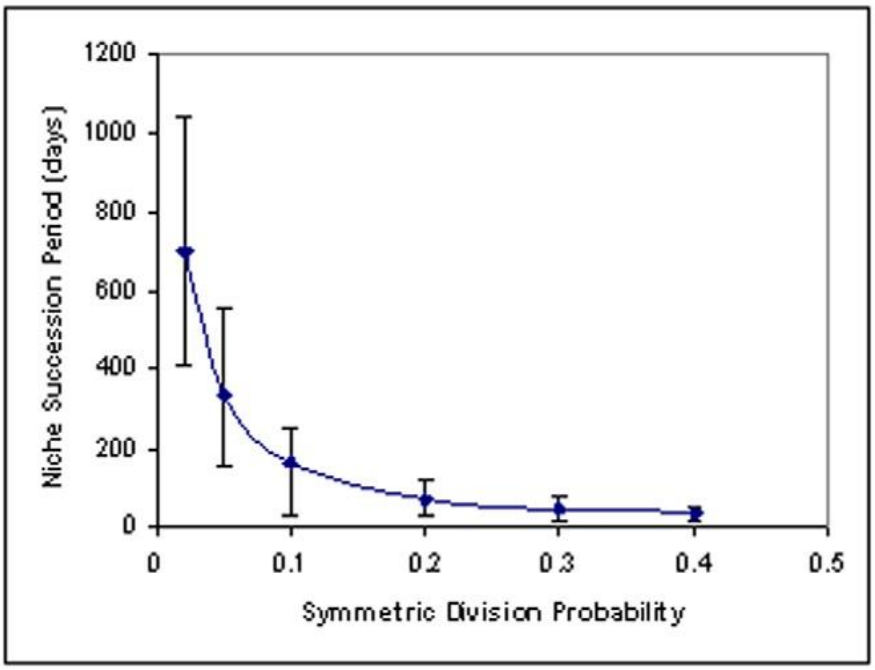


Figure 3.9 Variation of niche succession period with symmetric division probability.

3.4 Conclusions

With this model we are able to simulate the normal homeostatic processes in the crypt as well as follow stem cell lineages that establish themselves in the crypt by a population drift mechanism. The model can be utilized as a virtual experimental facility for testing different hypotheses and asking "what-if" questions. In the next chapter we report how we used the model to generate a hypothesis on the mechanism by which $APC^{+/-}$ mutation affects niche succession and colon cancer initiation.

4

Application of the Crypt Model - Explaining Precancerous Changes in FAP Crypts

In this chapter, we use the Agent-based model of the colon crypt (described in Chapter 3) to investigate the abnormalities of stem cell behavior that could be caused by mutation of the APC gene. Studies on individuals with Familial Adenomatous Polyposis (FAP) have shown that a single mutated APC allele can create changes in the precancerous colon crypt, like increased number of stem cells, increased crypt fission, greater variability of DNA methylation patterns, and higher somatic mutation rates. Comparing experimentally observed features of the crypts of FAP patients who harbor heterozygous $APC^{+/-}$ mutations with results from our computational experiments, we evolve a hypothesis on a mechanism by which $APC^{+/-}$mutation can affect the stem cell fate and set the stage for initiation of colon cancer. We find that our hypothesis can reproduce several observed features of the FAP crypt.

4.1 Biological background

Colorectal cancer arises as the cumulative effect of multiple mutations that enable the epithelial cell to escape all the controls that keep it from uncontrolled proliferation. Since in the colon mucosa, no cell other than the stem cells can survive more than a week, stem cells are the most reasonable candidates for the accumulation of multiple mutations. In this chapter we confine ourselves to studying the behavior of stem cells in the stem cell niche.

The initial genetic change in most colorectal adenomas is thought to be mutations in the tumor suppressor gene APC [370]. Mutations in APC can be identified in up to 80% of sporadic colorectal carcinomas. Individuals with heterozygous germline APC mutations as in Familial Adenomatous Polyposis (FAP) are born with normal appearing colons but hundreds of polyps start to appear during the second decade of life, suggesting that the

normal APC allele also needs to become dysfunctional for the tumor to progress. However there are indications [62] that even the normal appearing FAP colon crypts may be harboring morphologically occult changes introduced by heterozygous APC mutation though the mechanism of these changes is not clear.

APC is a multifunctional gene. It is a crucial member of the Wnt/β-catenin signaling pathway, which is an important determinant of cell proliferation, differentiation and apoptosis. APC also regulates cytoskeletal proteins including F-actin and microtubules, thus allowing it to regulate adhesion, migration and mitosis [370]. APC mutations generally result in truncated N-terminal protein fragments that cannot bind β-catenin and thus lose the function of Wnt/β-catenin regulation. Being a ‘‘loss of function’’ defect, heterozygous APC mutation is unlikely to have much effect on the Wnt/β-catenin signaling pathway. However it has been suggested that isolated N-terminal fragments can also have some ‘‘gain of function’’ effects on microtubules and spindle associated proteins in mitosis [236]. These effects can manifest even if only one APC allele is mutated.

Methylation patterns in normal appearing FAP crypts show greater diversity than non FAP crypts indicating slower niche succession cycles in FAP crypts. This slowing down can be explained as due to an increase in stem cell population. FAP crypts also exhibit a shift in the distribution of proliferative cells along the crypt axis and this also has been linked to an increase in stem cell number [362]. Increased crypt fission observed in FAP colons [20] is also indicative of increase of stem cell number [60]. Therefore it has been suggested that heterozygous APC mutations may be contributing to increase in stem cell number [62]. In addition, it has been observed that there is a 19 fold increase in the proportion of crypt fission in FAP colons [61].

In FAP colons the onset of cancer is actually faster than what could be expected if the pre-existing APC mutation implied only that the cells need one mutation less in order to initiate cancer [371]. Non-neoplastic FAP crypts are reported to have higher mutation rates [372, 373] which implies that heterozygous APC mutation has the effect of increasing mutation rates.

4.2 The question and the methodology

We start with the question, "What is the effect that a heterozygous APC mutation has on the behavior of an intestinal epithelial cell that can result in the changes observed in the precancerous FAP crypts?"

The methodology we follow is the following:

- Through computational experiments using an Agent-based model of colon crypt dynamics we evolve a hypothesis on how the mutation changes cell behavior.
- We incorporate this hypothesis of individual cell behavior into the model and investigate the consequences of the hypothesis in the overall behavior of the crypt.
- We check whether the changes in the crypt that occurred as a consequence of the hypothesis have actually been observed experimentally.

4.3 The computational model

The Agent-based model we developed for the colon crypt and the stem cell niche in the crypt is described in detail in Chapter 3. Our objective in this chapter is to evolve and test, through computational experiments, a hypothesis on the difference made by a heterozygous mutation of APC to the individual cell behavior. The hypothesis is then tested by seeing whether with this assumption on individual cell behavior, the observed changes in FAP crypts are shown up in the simulations. Therefore we extend the original model to include APC mutations.

4.4 Evolving a hypothesis on the effect of APC mutation

In Chapter 3 we studied how the probability of symmetric division changes the niche succession period which is the time period in which the stem cell niche is taken over by one of the stem cells and its progeny. The results from the study are once again reproduced here in Figure 4.1.

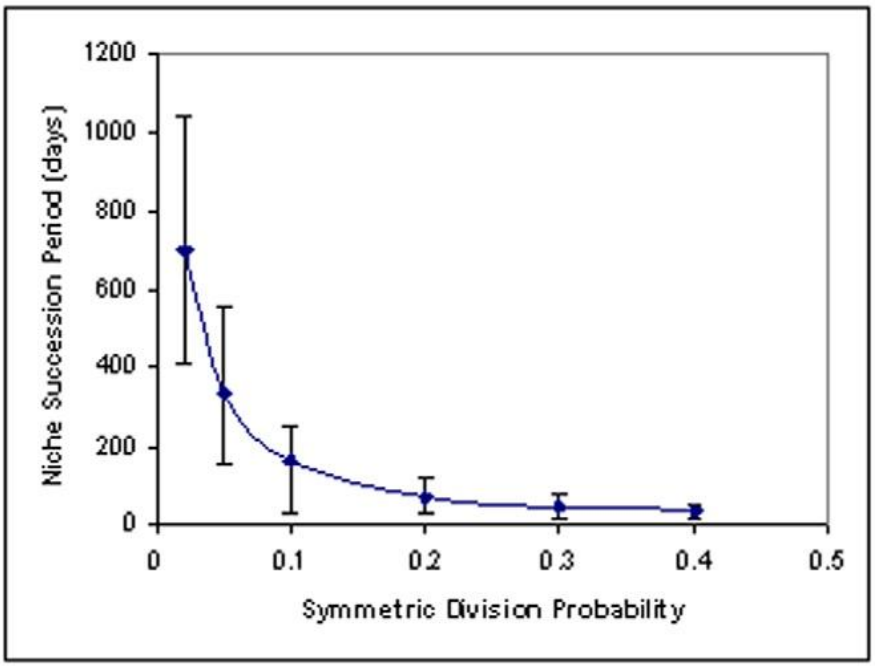


Figure 4.1 Variation of niche succession period with symmetric division probability

(For Description see Section 3.3.2 of Chapter 3)

There is an apparent contradiction between the results of Figure 4.1 and experimental observations on FAP crypts mentioned in Section 4.1:

- Figure 4.1 shows that niche succession period decreases when symmetric division increases.
- Methylation pattern analysis shows that niche succession period increases in APC mutated (FAP) crypts [62]. This implies that symmetric division probability is reduced in FAP crypts as per Figure 4.1.
- However it has been reported [15, 368] that APC mutation results in loss of asymmetric division in stem cells i.e. symmetric division probability increases in APC mutant stem cells.

We attempt to resolve this contradiction by hypothesizing that APC mutation not only increases symmetric division, but also biases the division in favor of producing stem cell progeny. In the normal crypt it is assumed that there are equal chances for differentiated progeny and stem cell progeny in symmetric division. If, in addition to increasing symmetric division, the APC mutation biases the symmetric division in favor of stem cell progeny, the stem cell number would increase and there is a likelihood that the larger number of stem cells would result in increasing the niche succession period.

The molecular mechanism of how heterozygous APC mutation increases and biases symmetric division is uncertain. Anchoring of stem cells in the niche appears to play an important role in the decision to divide symmetrically or asymmetrically [15, 365, 374] as well as the decision to differentiate or not [375, 376]. Whether the anchoring is related to APC control of the Wnt/β-catenin signaling pathway or whether it is through some other mechanism, has to be investigated. It has been suggested that haploinsufficiency in APC's control of the Wnt/β-catenin signaling pathway [62] results in accumulation of β-catenin which in turn affects adherens junctions between the stem cells and the niche and influences the cell's decision to differentiate or not [375, 376]. Another possibility is the effect of N-terminal fragments of mutated APC on microtubules and spindle associated proteins in mitosis [236].

4.5 Implementing the bias in favor of stem cell progeny in the model

The maintenance of stem cell number must be having an environmental control mechanism that gives global signals as to whether stem cell number needs to be increased or decreased.

The feedback corrected differentiation probability (Eqn. (3.4)):

$$P_d=0.5-(1-N_S/N_0), \tag{4.1}$$

that attempts to keep the stem cell number constant can be considered as a computational representation of the environmental control. The feedback corrected differentiation probability can take values in the range 0 to 1 depending on the number of stem cells at any point of time. Values of differentiation probability less than zero corresponding to $N_s/N_0<0.5$ are restricted to zero and values greater than one corresponding to $N_s/N_0>1.5$ are restricted to one.

APC mutation, while not having an impact on this environmental control, may still affect the response of the cell to the environmental control. The effect of APC mutation in biasing the division in favor of stem cell progeny can therefore be represented by reducing the feedback corrected differentiation probability by multiplying it by a biasing factor B less than 1. i.e.

$$P_d= B * (0.5- (1-N_S/N_0)) \tag{4.2}$$

$P_{2,}$ the probability of producing two stem cells (Eqn. (3.3)) and $P_{0,}$ the probability of producing no stem cells (Eqn. (3.1)) are in the same range when there is no bias (B= 1). The ranges of values possible for P_0 and P_2 for different bias factors are shown in Table 4.1. When the biasing factor is less than 1 it becomes possible for P_2 to have values greater than P_0. For example, for P_s = 0.2 and B =0.6, the biased differentiation probability can vary between 0 and 0.6 and P_0 can vary between 0 and 0.12 and P_2 can vary between 0.08 and 0.2. P_0 and P_2 have an overlapping range (0.08 to 0.12) outside of which P_2 can take values higher than P_0. When the biasing factor is less than 0.5, P_2 remains constantly larger than P_0.

Table 4.1 The ranges of values possible for P_0 and P_2 for different bias factors

Symmetric division probability (P_s)	Biasing factor for differentiation probability B	Range of biased differentiation probability (P_d)	Range of Probability of producing 0 stem cells $P_0=P_s P_d$	Range of probability of producing 2 stem cells $P_2=P_s(1-P_d)$
0.2	1	0 - 1	0 - 0.2	0 - 0.2
	0.8	0 - 0.8	0 - 0.16	0.04 - 0.2
	0.7	0 - 0.7	0 - 0.14	0.06 - 0.2
	0.6	0 - 0.6	0 - 0.12	0.08 - 0.2
	0.5	0 - 0.5	0 - 0.1	0.1- 0.2
	0.4	0 - 0.4	0 - 0.08 (always lower than P_2)	0.12 - 0.2 (always higher than P_0)
0.1	1	0 - 1	0 - 0.1	0 - 0 .1
	0.8	0 - 0.8	0 - 0.08	0.02 - 0 .1
	0.7	0 - 0.7	0 - 0.07	0.03 - 0 .1
	0.6	0 - 0.6	0 - 0.06	0.04 - 0 .1
	0.5	0 - 0.5	0 - 0.05	0.05 - 0 .1
	0.4	0 - 0.4	0 - 0.04 (always lower than P_2)	0.06 - 0.1 (always higher than P_0)
0.05	1	0 - 1	0 - 0.05	0 - 0 .05
	0.8	0 - 0.8	0 - 0.04	0.01 - 0.05
	0.7	0 - 0.7	0 - 0.035	0.015 - 0 .05
	0.6	0 - 0.6	0 - 0.03	0.02 - 0 .05
	0.5	0 - 0.5	0 - 0.025	0.025 - 0.05
	0.4	0 - 0.4	0 - 0.02 (always lower than P_2)	0.03 - 0 .05 (always higher than P_0)
0.02	1	0 - 1	0 - 0 .02	0 - 0 .02
	0.8	0 - 0.8	0 - 0.016	0.004 - 0.02
	0.7	0 - 0.7	0 - 0.014	0.006 - 0.02
	0.6	0 - 0.6	0 - 0 .12	0.008 - 0.02
	0.5	0 - 0.5	0 - 0.01	0.01 - 0 .02
	0.4	0 - 0.4	0 - 0.008 (always lower than P_2)	0.012 - 0 .02 (always higher than P_0)

4.6 Testing the hypothesis - Can the hypothesis resolve the contradiction?

Simulations of stem cell dynamics in the niche were performed with biased differentiation probability under different values for the bias factor B.

Figures 4.2 (A–C) shows the variation of stem cell number with time (iteration number) for different values of the biasing factor. When the biasing factor equals 1 and P_0 and P_2 are in the same range the number of stem cells is maintained within a range 8–13 with an average value of 10 (Figure 4.2A). For values of the biasing factor between 1 and 0.5, the ranges of P_2 and P_0 are such that P_2 can assume values greater than P_0 and therefore the average stem cell number increases but the correction factor still controls the stem cell number from inordinate increase (Figure 4.2B). When the biasing factor is made less than 0.5 the stem cell number increases uncontrollably (Figure 4.2C) because P_2 is always larger than P_0.

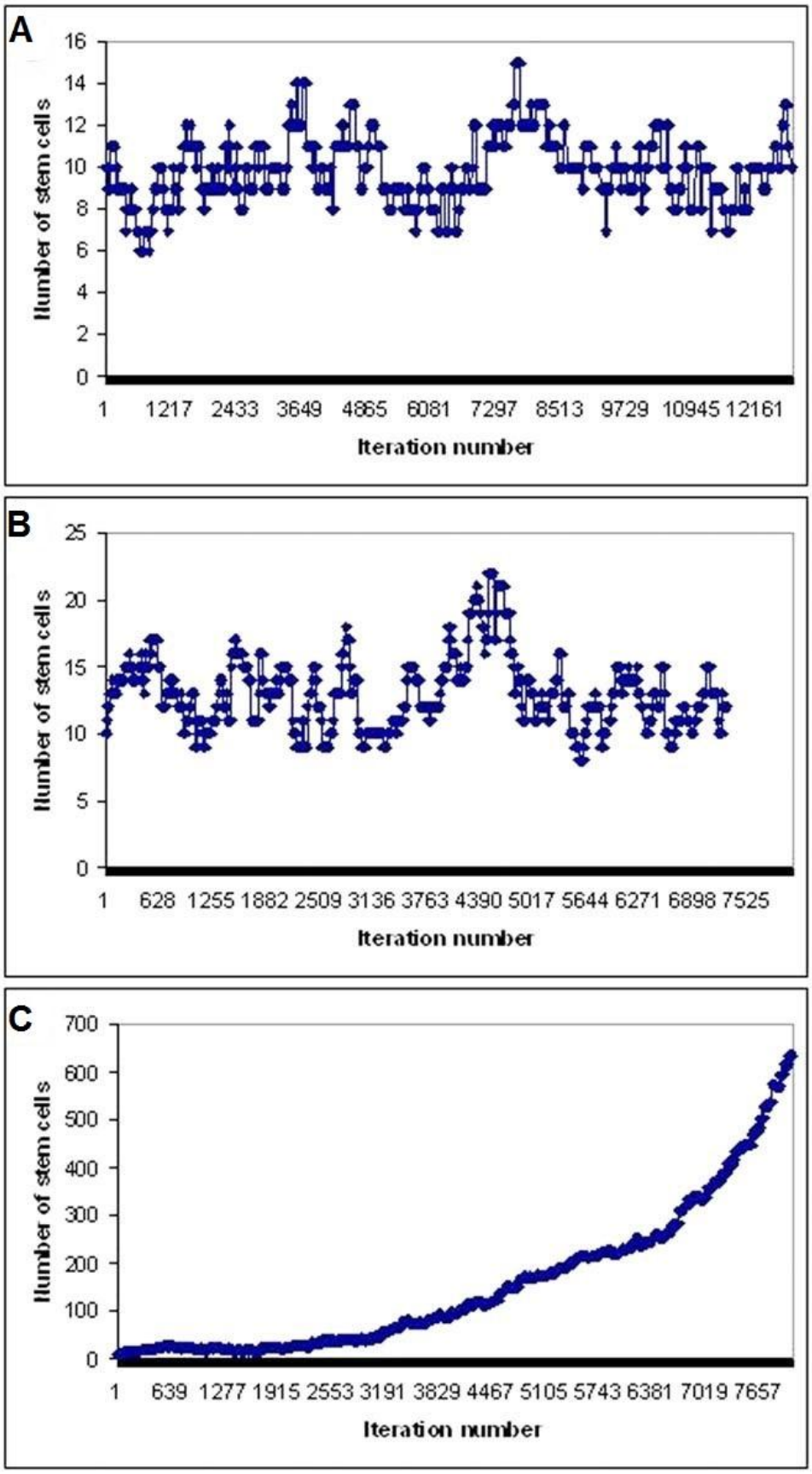


Figure 4.2 Time variation of stem cell number with: (A) Bias factor = 1, (B) Bias factor = 0.5, (C) Bias factor = 0.4.

Table 4.2 Stem cell number and niche succession period for different values of symmetric division probability, P_s and the biasing factor, B.

Symmetric division probability (P_s)	Biasing factor for differentiation probability B	Average stem cell number	Range of stem cell number (between 5th and 95th percentile)	Average Niche succession period (days)
0.2	1	10	8 -13	60
	0.8	11	9 -14	66
	0.7	12	10 -16	78
	0.6	13	10 -18	70
	0.5	15	11 - 20	99
	0.4	Continuous increase	~	More than one lineage survives
0.1	1	10	8 -13	100
	0.8	11	9 -14	120
	0.7	12	11 -16	156
	0.6	13	11 -18	215
	0.5	32	13 - 83	374
	0.4	Continuous increase	~	More than one lineage survives
0.05	1	10	8–13	339
	0.8	11	9–15	376
	0.7	12	9–16	428
	0.6	13	12–20	458
	0.5	55	14–132	862
	0.4	Continuous increase	~	More than one lineage survives
0.02	1	10	7–13	697
	0.8	11	8–14	829
	0.7	12	7–15	920
	0.6	13	9 –17	1234
	0.5	31	13–68	2339
	0.4	Continuous increase	~	More than one lineage survives

Table 4.2 shows the stem cell number and niche succession period for different values of symmetric division probability, P_s and the biasing factor, B. Change of symmetric division probability is seen to have no effect on the stem cell number as long as there is

no bias in the differentiation probability. The niche succession period is affected by both symmetric division probability and differentiation probability. For the same differentiation probability the niche succession period decreases as the symmetric division probability increases. For the same symmetric division probability the niche succession period increases as the biasing factor decreases. If the differentiation probability decreases along with increase of symmetric division probability the niche succession period can increase only in certain ranges of the parameters. For example, when symmetric division probability increases from 0.02 to 0.05 and at the same time the biasing factor for differentiation probability decreases from 1 to 0.5 the niche succession period increases from 697 days to 812 days.

However on the whole the effect of the biasing factor is not very dramatic as long as it is above 0.5. When the biasing factor goes below 0.5 the simulations show uncontrolled increase of stem cell number because P_2 is always more than P_0 (Table 4.2). This would correspond to a situation where the mutated stem cells completely ignore the environmental signal to produce differentiated progeny. The large number of stem cells ensures that two or three lineages continue to persist and so complete niche take over by a single lineage gets delayed indefinitely. In fact we found that computer memory overflow occurs before niche succession could be observed. In real crypts it would be impossible for the niche to contain such large number of stem cells and the pressure of overcrowding would probably lead to crypt fission (Figure 4.3) which is not dealt with in this model, but has been observed experimentally in FAP crypts.

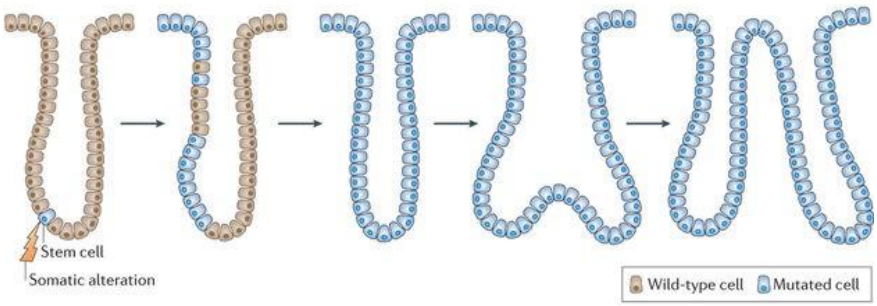


Figure 4.3 Crypt fission. (Reproduced from Curtius et al. (2018)) [377])

Thus the hypothesis that heterozygous APC mutation not only increases symmetric division probability but also biases symmetric division towards producing stem cell progeny can produce consequences consistent with the following observations from previous studies:

- Heterozygous APC mutation increases symmetric division in stem cells [15, 368].
- Precancerous FAP crypts show increased niche succession period [62].
- Stem cell number is increased in FAP crypts [20, 60, 62, 362].
- Crypt fission is also increased in FAP crypts [20].

On the other hand if APC mutation only increases symmetric division, the niche succession period would decrease and there would be no increase in stem cell number. Therefore the bias in favor of stem cell progeny is essential to explain the observed changes in FAP crypts.

In the following sections we show that if our hypothesis is true, i.e. if heterozygous APC mutation has the effect of increasing as well as biasing symmetric division, cells with this mutation have increased probability for initiating colorectal cancer.

4.7 Implementing APC mutation in the model

Every cell agent is assumed to possess two copies of the APC gene which can be in a mutated or un-mutated state. We add two attributes “Mutation State1” and “Mutation State2” to represent the mutation states of the two APC alleles of the cell agent. The attributes can take values 0 or 1 representing un-mutated and mutated states respectively. For modeling FAP all the cells are assumed to have a mutation in one copy of the APC gene. This is represented by setting the initial values of Mutation State1 as 1 and Mutation State2 as 0 for all cell agents. For modeling sporadic mutation the simulation begins at a point when one of the stem cells has acquired a mutation in one of the APC alleles. This is represented by setting initial values of Mutation State1 to 1 and Mutation State2 to 0 for one of the stem cell agents.

Somatic mutation of the second APC allele is assumed to take place with a probability defined by an input parameter ‘‘Mutation probability’’ which defines the probability that the APC gene gets an additional mutation during the division of a cell. When the mutation probability is greater than a random number generated during division of the cell, the second allele is assumed to become mutated and Mutation State2 is set to 1 for the cell.

The change in behavior of a cell with a mutated APC gene is implemented as a new rule that implements our hypothesis that a mutated stem cell has a higher probability for symmetric division and that symmetric division is biased in favor of stem cell progeny.

4.7.1 Sporadic mutation that increases biased symmetric division has a better chance of getting fixed in the crypt

Previous theoretical studies indicate that symmetric division (and consequent niche succession) protects the cell from accumulation of mutations [342, 371] because a stem cell lineage bearing one mutation "has more chance of becoming extinct during the next niche succession cycle than of benefiting from the advantages of fixation in the crypt". However these results are valid only if the mutation is neutral from the point of view of niche succession. If, on the other hand, the mutation has the effect of giving a selective advantage for niche succession, the mutation would be able to ‘‘hitch-hike’’ on niche succession and get fixed in the crypt.

The observation that most sporadic colon cancers are initiated by APC mutation suggests that the first APC mutation has a better chance than any other mutation for getting fixed in the crypt by niche succession. We show below that if the effect of heterozygous APC mutation is only to increase symmetric division, an APC mutated stem cell lineage has a higher chance of getting extinct. On the other hand if the effect of the mutation is not only to increase symmetric division, but also to bias it in favor of stem cell progeny, we show below that the mutation has a high probability of getting fixed in the crypt.

We performed simulations where 9 out of the 10 stem cells were considered normal (control) cells, and one stem cell was assumed to have a mutation that makes its symmetric division probability twice the control probability and a differentiation probability that is biased to different degrees in favor of producing stem cell progeny. The control symmetric division probability is assumed to be 0.1. The results are shown in Table 4.3.

Table 4.3 Probability of niche succession assuming different effects for the $APC^{+/-}$ mutation.

Effect of mutation	Probability of niche succession by the mutated cell
No effect	10%
Symmetric division probability is doubled but unbiased	3%
Symmetric division probability is doubled and biased, B = 0.6	45%
Symmetric division probability is doubled and biased, B = 0.5	75%
Rate of division is doubled	0%

A mutation that enhances biased symmetric division increases the probability of fixation significantly above the random (one in ten) probability of 10%, the probability increasing as the division gets more and more biased in favor of stem cell progeny. On the other hand increase of symmetric division without the bias decreases the probability of fixation to below the random probability in agreement with previous theoretical studies [342, 371].

Interestingly, when it was assumed that the effect of the mutation is to increase the rate of division by a factor of 2 the mutated cell lineage was lost in all the simulations because the increased number of divisions only increases the chances of its extinction. Therefore a proliferative advantage actually seems to work against the survival of the stem cell lineage. It is thus likely that if our hypothesis is true, a sporadic heterozygous APC mutation gets fixed in the crypt before the acquisition of proliferative mutations. This fits very well with the observation that most sporadic colorectal cancers are initiated by APC mutation even though there are other mutations like in K-ras or β-catenin that could as well enhance cell proliferation.

4.7.2 Mutation that increases biased symmetric division increases somatic mutation rates

FAP colon cells already harbor one potentially carcinogenic mutation. Since the onset of cancer requires a series of mutations, the FAP cells require one mutation less for the onset of cancer and therefore can be expected to proceed to cancer a little faster than cells with no inherited carcinogenic mutations. But FAP colons are actually seen to proceed to cancer faster than what could be expected from the simple advantage of

having one mutation less to acquire in the journey to cancer [371]. The heterozygous APC mutation therefore seems to confer some advantage that allows it to acquire the rest of the needed mutations faster. In fact, non-neoplastic FAP crypts are reported to have higher mutation rates [372, 373] which implies that heterozygous APC mutation has the effect of increasing mutation rates.

An interesting computational study by Pepper et al. [378] shows that serial differentiation is the key to suppression of somatic mutation in multicellular tissues. Asymmetric division as well as self-renewing symmetric division increase somatic mutation rate in a tissue by increasing the number of cells in the proliferating pool. In our model transit amplifying cells undergo serial differentiation by always dividing symmetrically into further differentiated progeny. The stem cells on the other hand have the capability for all the three modes of division viz. asymmetric, self-renewing symmetric and differentiated symmetric. Table 4.4 shows the iteration number at which the somatic mutation of the second APC allele takes place for different values of symmetric division probability and differentiation probability. The mutation probability of the normal APC allele is assumed to be 0.001 and normal symmetric division probability is set to 0.1. APC mutation is assumed to increase the symmetric division probability to 0.2 and bias it by a bias factor of 0.5. The mutation rate used here does not have any real basis and a high value is used in order to speed up the computation time. Our aim in performing these experiments is only to see the relative effects that symmetric division probability and differentiation probability have on the time for acquiring a second mutation and therefore actual values of the times are not important in these investigations.

Table 4.4 The effect of symmetric division probability and differentiation probability on the time for acquiring a second mutation.

Effect of heterozygous APC mutation	Symmetric division probability	Bias factor for differentiation probability	Average number of iterations to the second mutation
No effect	0.1	1	6460
Increased symmetric division	0.2	1	6220
Increased biased symmetric division	0.2	0.5	3939

We see that increased symmetric division by itself does not have a significant effect on the time taken for the second mutation. When the symmetric division is biased in favor of stem cell progeny the time for appearance of the second mutation is reduced. Thus the hypothesis that heterozygous APC mutation that results in biased symmetric division is able to explain the increase of somatic mutation rates in FAP crypts.

4.8 Conclusions

In summary, the hypothesis that heterozygous APC mutation increases symmetric division of stem cells as well as biases the division in favor of stem cell progeny, results in the following effects:

1. FAP crypts have increased stem cell numbers and increased niche succession period.
2. Sporadic APC mutation has a high probability of getting fixed in the crypt by niche succession. On the other hand a mutation that increases proliferation rate increases the chance of extinction of the stem cell lineage.
3. Somatic mutation rates are increased in cells which have APC mutation.

Thus, through getting dominance in the crypt, through increase in stem cell number and consequent crypt fission, and increased chances of replication errors, heterozygous APC mutation sets the stage for colorectal cancer. If the molecular mechanism by which the mutant APC effects the increase of biased symmetric division can be experimentally deciphered it can lead to drugs that could prevent the progression to cancer in people who have inherited or acquired a mutation in one of their APC alleles.

5

Multiple Roles of Adenomatous Polyposis Coli Gene in Wnt Signaling – a Computational Model.

In this chapter we go one level deeper to the level of molecular interactions. We take a look at the multiple roles played by APC in the most highly implicated pathway in Colon cancer, namely the Wnt/β-catenin signaling pathway. We present here a computational model for the Wnt/β-catenin signaling pathway that explicitly includes the five different roles of APC in regulating β-catenin/TCF formation:

1. APC is part of the Destruction Complex that phosphorylates cytoplasmic β-catenin in a way that marks it for destruction.
2. APC retains cytoplasmic β-catenin by binding to it.
3. APC plays a role in controlling the distribution of β-catenin between Wnt signaling and cell-cell adhesion.
4. APC translocates to the nucleus and competes with TCF for binding to β-catenin within the nucleus.
5. APC promotes the export of β-catenin out of the nucleus.

We use this computational model to perform in-silico experiments to study the effect of different functional losses of APC on the level of β-catenin/TCF complex. The simulations also demonstrate the different outcomes that could be expected when the system is governed by different hypotheses regarding the underlying phenomena.

5.1 Biological background

The Adenomatous Polyposis Coli (APC) gene is found to be mutated in 80% of colon cancers indicating that this gene is a key regulator of cell division in the colon [379]. The Canonical Wnt pathway plays a major role in the self renewal process of continuously renewed tissues like the colon crypt and APC has been found to be a negative regulator of this pathway [283]. APC regulates the Wnt/β-catenin signaling pathway by controlling the formation of β-catenin/TCF, a nuclear complex of the transcriptional

effector protein β-catenin with T cell factor (TCF). The repression of TCF-mediated transcription by APC is thought to be the main basis for its function in normal development and in preventing tumorigenesis [380] .

5.1.1The mechanisms by which APC can control β-catenin/TCF formation

The most well-known mechanism, by which APC controls β-catenin/TCF formation, is by the maintenance of a low level of β-catenin in the cytoplasm so that probability of its nuclear translocation is reduced. APC is known to be a critical component of a Destruction Complex that phosphorylates cytoplasmic β-catenin and marks it for ubiquitination and subsequent degradation [107, 134, 144, 296, 381] . When Wnt signals bind to cell surface receptors, the Destruction Complex becomes dysfunctional. The cytoplasmic level of β-catenin increases and it translocates to the nucleus and binds with TCF. This initiates transcription of the Wnt target genes and consequent cell division. Certain mutations of APC also can render the Destruction Complex ineffective and this can lead to uncontrolled cell division even in the absence of Wnt signals. Sequestration of β-catenin by APC is also another mechanism by which APC can lower the level of free β-catenin in the cytoplasm.

β-catenin has other important functions apart from transmitting the Wnt signal for initiation of cell division. β-catenin participates in cell-cell adherence junctions by binding to E-cadherin. β-catenin binds to the cytoplasmic domain of E-cadherin which, together with the actin-binding protein, α-catenin, allows cells to link their cytoskeletal networks through intercellular adherence junctions. It has been observed that APC affects cell adhesion by controlling the distributions of β-catenin between Wnt signaling and cadherin binding [168, 360, 382]. However the mechanism of control is unclear. One possibility is that β-catenin bound to APC is transported to the membrane thus facilitating its binding to E-cadherin [96]. Another possibility is that binding to APC results in a form of β-catenin that prefers binding to E-cadherin over translocation to the nucleus. It has been observed that the switch between β-catenin's adhesive and nuclear translocation functions is modulated by phosphorylation of Tyrosine 142 (Y142) residue of β-catenin [294] , which favours its binding to the nuclear transporter Bcl9 and disfavours its binding to E-cadherin. It could be postulated that APC plays a role in decreasing the level of Y142 phosphorylated β-catenin.

The APC protein has been found to be capable of shuttling between the cytoplasm and the nucleus [112] . In the nucleus, its binding affinity for β-catenin can downregulate the formation of the β-catenin/TCF complex. It has also been suggested that APC significantly enhances the export of β-catenin out of the nucleus [112, 140], again decreasing the probability for β-catenin/TCF complex formation.

In summary, APC can down-regulate the formation of β-catenin/TCF complex through at least 5 mechanisms viz. (1) reducing β-catenin concentration in the cytoplasm by facilitating its destruction, (2) by sequestration of cytoplasmic β-catenin, (3) by influencing β-catenin to bind to E-cadherin instead getting transported to the nucleus, (4) by providing a competition to TCF for binding to nuclear β-catenin and (5) by promoting export of β-catenin out of the nucleus. In this chapter we present a computational model of the Wnt/β-catenin signaling pathway that includes all these five regulatory roles of APC. We use the model to study the effect of different functional losses of APC on the formation of β-catenin/TCF complex.

5.1.2 Mutation of APC and consequent functional losses.

Mutation in the APC gene is the basis of inherited predisposition to colorectal cancer in FAP and is also the primary event in the initiation of sporadic colorectal tumors [383, 384]. Some mutations result in complete loss of the APC protein while others result in truncated forms of the protein. Different mutations affect the various roles that APC plays in the Wnt/β-catenin signaling pathway differently.

The regulation of β-catenin activity by APC depends primarily on its ability to bind β-catenin. APC protein contains two separated domains capable of interacting with β-catenin. The first domain consists of three 15aa repeats, whereas the second domain contains seven 20aa repeats [384]. Binding of β-catenin to the 15aa repeats on APC does not mark β-catenin for subsequent degradation unlike binding at the 20aa repeats [384]. When APC lacks active 20aa sites, it no longer contributes much to degradation of β-catenin, but it can still sequester β-catenin in the cytoplasm and nucleus by binding it to the 15aa sites.

In addition to the β-catenin binding sites, other sites can be involved in β-catenin regulation by APC and mutation of these can also affect Wnt signaling in different ways. The formation of the Destruction Complex depends on APC binding to Axin through

short “SAMP” motifs and therefore absence of SAMP would disrupt the formation of the Destruction Complex [384]. The shuttling of APC in and out of the nucleus involves several nuclear localization signals (NLS) and nuclear export signals (NES) found on the APC protein. APC lacking in NLS cannot translocate to the nucleus and contribute to nuclear sequestration and export of β-catenin. APC lacking in NES may not be able to perform the role of enhancing the export of β-catenin out of the nucleus [95, 112, 140-142, 385, 386]. Since our model explicitly considers the different roles of APC in Wnt signaling, it is possible to study the effect of different functional losses of APC using the model.

5.2 Computational models for the Wnt signaling network.

Over the years several models have been developed for studying the dynamics of the Wnt/β-catenin signaling pathway [387, 388]. The first quantitative model of the canonical Wnt/β-catenin signaling pathway was proposed by Lee et al. [292]. They developed a system of coupled ODE's to describe how the key proteins in the pathway change over time in response to the Wnt stimulation. Several models followed, that further analysed this model [389-392], or modified and extended the model to include feedback loops [391, 393], mutations of the pathway components [393-395], interaction with other networks [396] and cytoplasmic/nuclear shuttling of APC [397, 398]. Most of these followed the Lee et al. [292] model for the formation of the Destruction Complex, and its Wnt induced inhibition. Also the steps of the core destruction cycle were also inherited as such from the Lee et al. [292] model.

In these models, the Destruction Complex is formed by the binding of Glycogen Synthase Kinase 3β (GSK3β) to APC/Axin complex. The destruction cycle consists of four steps viz. Phosphorylation of the Destruction Complex, binding of β-catenin to the phosphorylated Destruction Complex, phosphorylation of β-catenin, and its release from the Destruction Complex for proteolysis. When the Wnt ligands bind to the cell surface receptors, GSK3β is dissociated from the Destruction Complex causing disassembly of the Destruction Complex.

Later experimental findings have questioned the assumption of disassembly of the Destruction Complex by Wnt signaling [311]. Chen et al. [399] modified the basic Lee et al. [292] model for the destruction cycle to account for later experimental findings [400-

403]. Mainly the assumption of total inhibition of the Destruction Complex by Wnt signaling was modified to account for the observation that the Destruction Complex remains intact under Wnt stimulation. Also the importance of phosphorylation in regulating the distribution of β-catenin between cytoplasmic, nuclear and membrane pools was taken into account. With these modifications, the kinetic response of phosphorylated and non phosphorylated β-catenin to Wnt signaling could be qualitatively reproduced by the Chen et al. [399] model. The focus of the Chen et al. [399] model was on the interplay between Wnt signaling and Cadherin mediated cell adhesion; therefore the model also incorporated a detailed model of cell adhesion as well as a transcription model that feeds back to the cell adhesion model.

Our focus in this work is the role of APC in Wnt signaling and the consequences of APC mutation on the level of β-catenin/TCF, which is an indicator of the level of consequent cell proliferation. Our strategy is to build into an existing Wnt Signaling model, additional features that help us study the multiple ways in which APC acts to control the level of β-catenin/TCF. We have chosen to use the Chen et al. [399] model for Wnt signaling as our base model because it incorporates later experimental findings which are not considered in the Lee et al. [292] model. These are elaborated in the next section. The Chen et al. [399] model does not explicitly consider the role of APC in the destruction cycle or in the E-cadherin binding of β-catenin. It also does not consider the sequestering and export of nuclear β-catenin by APC. Some of these roles have been modeled separately in other papers [292, 360, 397, 398]. In this chapter, based on literature reports, we put together conceptual models for five different roles played by APC in regulation of the Wnt/β-catenin signaling pathway. Our methodology is to incorporate these models into the basic Wnt signaling model inherited from the Chen et al. [399] model.

5.3 Methodology

Since the heart of our model is the Wnt signaling model inherited from Chen et al. [399], we describe here the essential differences of this model from the Lee et al. [292] model that forms the basis for most of the existing models. Specifically, the Chen et al.[399] model differs from the Lee et al. [292] model on the following points:

Wnt stimulation does not result in disassembly of the Destruction Complex. It has been experimentally observed by Li et al. [311] that the Destruction Complex remains intact under Wnt stimulation. The Chen et al. [399] model assumes that when Wnt is "On", the majority of Destruction Complexes are recruited onto membrane proximal regions and bind to Wnt receptors. So, under Wnt stimulation, there are two simultaneous destruction cycles, one in cytoplasm and one at the membrane, but the cycle at the membrane becomes the major pathway for β-catenin destruction.

The phosphorylation of β-catenin by the Destruction Complex takes place sequentially and not in a single step. β-catenin, on binding to the Destruction Complex, is first phosphorylated at residue S45 by Casein Kinase 1 (CK1) and this primes it for further phosphorylation by GSK3β at residues S33/37/T41 [294].

After phosphorylation by GSK3β, the Destruction Complex is assumed to undergo a structural transformation that exposes β-catenins to ubiquitin/proteasome machinery for degradation. Once the β-catenins are degraded, the Destruction Complex resumes its original state and binds more β-catenin [295].

It is only the "Active β-catenin" (ABC), which is β-catenin that is phosphorylated at Serine 45 (S45) and unphosphorylated at S33/37 that accumulates in the nucleus and triggers Wnt target gene expressions [404]. The CK1-phosphorylated β-catenin that gets released from the Destruction Cycle is assumed to be ABC [404] and only these are allowed to translocate to the nucleus and bind to TCF. It is also assumed that under Wnt stimulation, the binding of the Destruction Complex to the membrane receptors enhances structural changes that facilitate the release of ABC at the stage of CK1 phosphorylation.

Translocation to nucleus is assumed to need an additional phosphorylation at Y142 residue by a Tyrosine Kinase in order to bind to the nuclear transporter Bcl9. Phosphorylation at Y142 residue has been found to be a functional switch for β-catenins between cell adhesion and Wnt signaling [313, 314].

The computational model of Chen et al. [399] was set up as a stochastic simulation model solved using the Gillespie algorithm instead of the deterministic ODE formulation followed in the Lee et al. [292] model. This formulation accounts for the stochastic nature of molecular processes in biological cells. Single cell protein concentrations were

converted to number of molecules. A subcellular system that occupies 2.5% volume of a whole cell (assumed to be 10-12 litre) was simulated.

We inherit the Wnt signaling model from Chet et al. [399] and incorporate into it, our models for the five roles played by APC in the Wnt/β-catenin signaling pathway. Following the computational approach of Chen et al.[399], our model is also set up as a stochastic simulation model and numerically solved using the Gillespie algorithm [332].

The Chen et al. [399] model, of the Wnt/β-catenin signaling pathway is reproduced from their paper [399] as Figure 5.1. The abbreviations in the model are given in Table 5.1. We incorporate our models for the roles played by APC into this model.

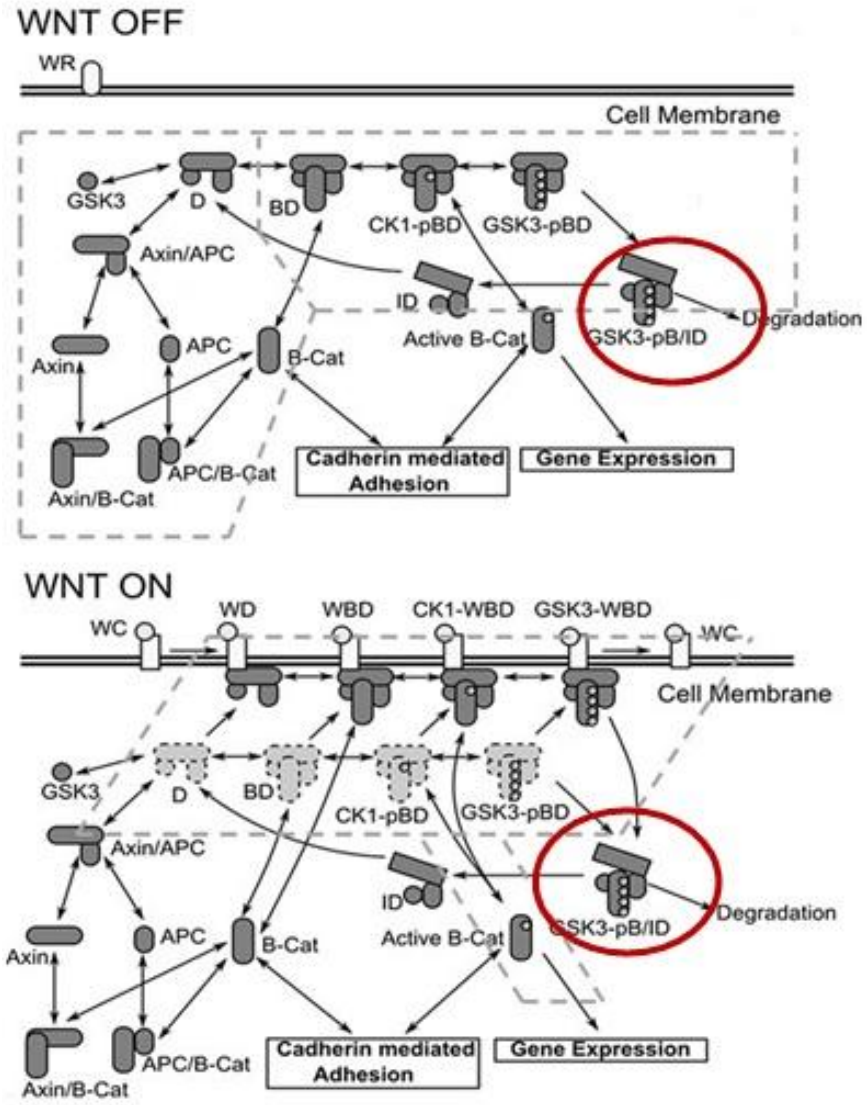


Figure 5.1 Chen et al. [399] model for destruction cycle under Wnt "Off" and Wnt "On" conditions.
The degradation step for β-catenin (circled) as implemented in our model is elaborated in Figure 5.2.

Table 5.1.Expansion of abbreviations in Figure 5.1.

Abbreviation	Expansion
APC	Adenomatous Polyposis Coli gene.
Axin	Axin
Axin/APC	Axin/APC Complex
CK1	Casein Kinase 1
GSK3β	Glycogen Synthase Kinase (GSK3β)
D	Destruction Complex
BD	β-catenin bound to D
CK1-pBD	CK1 phosphorylated β-catenin bound to D
GSK3-pBD	GSk3β phosphorylated β-catenin bound to D
GSK3-pB/ID	GSk3β phosphorylated β-catenin bound to Inactive D
ID	Inactive Destruction Complex
ABC	Active β-catenin (β-catenin phosphorylated at Serine 45)
pABC	ABC phosphorylated at Y142
WC	Wnt Receptor Complex
WD	Destruction Complex bound to Wnt
WBD	β-catenin bound to WD
CK1-WBD	Phosphorylated β-catenin bound to WD
GSK3-WBD	GSk3β phosphorylated β-catenin bound to WD
Axin/B-Cat	β-catenin bound to Axin
APC/B-Cat	β-catenin bound to APC

When there is no Wnt signal there is no formation of the Wnt Receptor Complex WC, no binding of Destruction complex to the Wnt receptor Complex and therefore no formation of WD or WBD. Wnt OFF condition is modeled by assigning value of zero to the reaction constants for the formation of all complexes with the Wnt receptor.

The conceptual models that we use for studying the roles played by APC are as follows:

5.3.1 Model for the role of APC in destruction cycle.

The Chen et al. [399] model assumes that after GSK3β phosphorylation of β-catenin, the Destruction Complex undergoes a structural transformation that exposes β-catenin to the ubiquitin/proteasome machinery for degradation. They do not explicitly consider the role of APC in this process. In order to model the role of APC and the effect of its mutation we need to go into more details of the destruction cycle.

In the Destruction Complex, it is Axin that plays the role of the scaffold for β-catenin [107]. The role of APC in the destruction cycle is still unclear. It has been proposed [134] that after phosphorylating β-catenin, CK1 and GSK3β phosphorylate APC; phosphorylation of the APC 20aa repeats enhances their affinity for β-catenin[299] considerably over that of Axin and β-catenin shifts from Axin to APC. This causes dephosphorylation of Axin and inactivation of the Destruction Complex and it gets structurally transformed in a way that exposes β-catenin for degradation [294]. Therefore in our model, we assume that it is the binding of β-catenin to APC is the critical step that causes the transformation which exposes β-catenin for degradation. If APC is mutated such that the 20aa repeats are inactive or absent, the transformation step does not happen. We show below the destruction cycle steps of Chen et al. [399] model along with our model of the molecular events that form the basis for these steps:

Step 1 - GSK3-pBD ⟶ GSK3-pB/ID

Molecular events:

- APC 20aa sites are phosphorylated.
- β-catenin shifts from Axin to APC and the APC-bound β-catenin is exposed to the degradation machinery .
- Axin is dephosphorylated and the Destruction Complex is inactivated.

Step 2 - GSK3-pB/ID ⟶ Degraded β-catenin + ID

Molecular event:

- β-catenin is degraded and leaves behind the Inactive Destruction Complex.

Step 3 - ID ⟶ D

Molecular event:

- Axin gets phosphorylated and the Destruction Complex is active again

In our model, binding to APC is the critical step in exposing β-catenin for degradation. Effect of the 20aa mutation of APC can be modeled therefore by reducing the probability of the transition from GSK3-pBD ⟶GSK3-pB/ID (Step 1). The scheme is summarized in Figure 5.2.

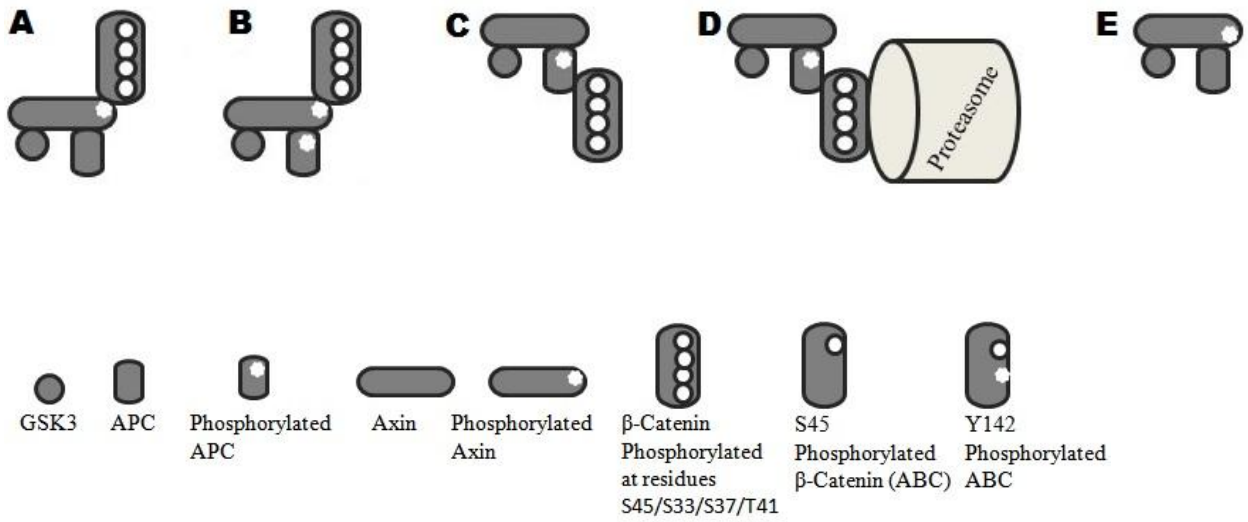


Figure 5.2 The elaborated model for the degradation of β-catenin assumed in this work. (A) β-catenin which is bound to phosphorylated Axin on the Destruction Complex is phosphorylated by GSK3. (B) APC is phosphorylated by GSK3. (C) β-catenin shifts from Axin to APC. Axin is de-phosphorylated and the Destruction Complex is structurally transformed to an inactive state. (D) APC-bound β-catenin is handed over to the Proteasome Machinery for degradation. (E) Axin gets phosphorylated again and the Destruction Complex becomes active once more.

5.3.2 Model for the role of APC in controlling the distribution of β-catenin between adhesion and Wnt signaling.

Cytoplasmic β-catenins have several binding choices: bind with E-cadherin at the cell membrane to form Cad/Cat complexes, bind with the nuclear transporter Bcl9, bind to the Destruction Complex, or bind to free APC or Axin. The state of phosphorylation of β-catenin plays a role in these choices. Our model makes the following assumptions based on the Chen et al. [399] model and experimental observations:

1. Unphosphorylated β-catenin can bind to E-cadherin, APC and to the Axin in the Destruction Complex [399].
2. Active β-catenin (ABC), phosphorylated at Serine 45, released from the Destruction Complex at the stage of CK1 phosphorylation can bind to E-Cadherin and APC [399].

3. Only ABC which is further phosphorylated at the Y142 residue (pABC) can bind to nuclear transporter Bcl9 and translocate to the nucleus and initiate transcription [313, 399].
4. Y142-phosphorylated ABC (pABC) cannot bind to E-cadherin [405].

We propose a hypothesis for APC's role in directing β-catenin towards the membrane and binding to E-cadherin:

Since Y142 phosphorylation seems to be the critical step that makes ABC prefer binding to nuclear transporter Bcl9 over binding to E-Cadherin, APC may be playing a role in reducing the level of Y142 phosphorylated ABC in the cytoplasm. We speculate that Y142-phosphorylated ABC (pABC) that binds to APC gets released from APC as ABC (unphosphorylated at Y142) and this ABC binds to E-cadherin in preference to Bcl9 (Figure 5.3).

APC + pABC ⟶ APC/pABC

APC/pABC ⟶ APC + ABC

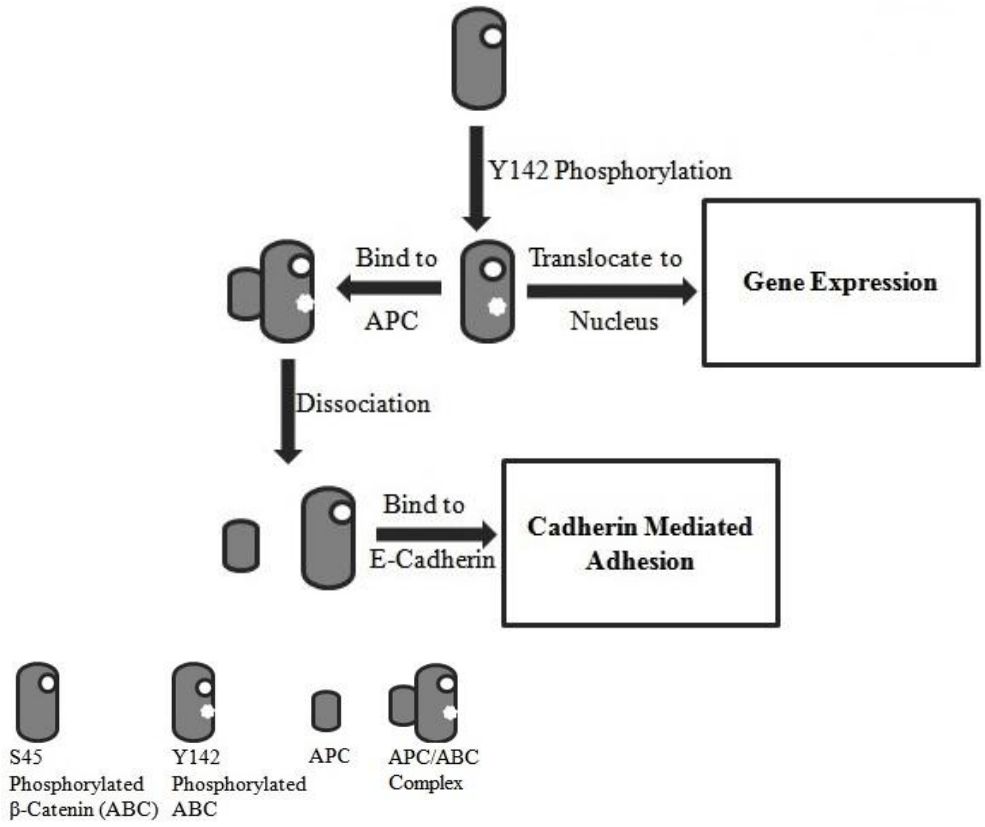


Figure 5.3 Conceptual model for how APC plays a part in controlling the distribution of β-catenin between gene expression and E-cadherin mediated adhesion.

5.3.3 Sequestration and export of ABC by nuclear APC.

APC contains conserved nuclear export signals and are capable of shuttling between cytoplasm and the nucleus. Nuclear APC binds to nuclear β-catenin (pABC) thereby decreasing the pool of β-catenin for transcription activation.

We assume that APC contributes to β-catenin export by the export of the APC/ABC complex from the nucleus [112, 140] (Figure 5.4).

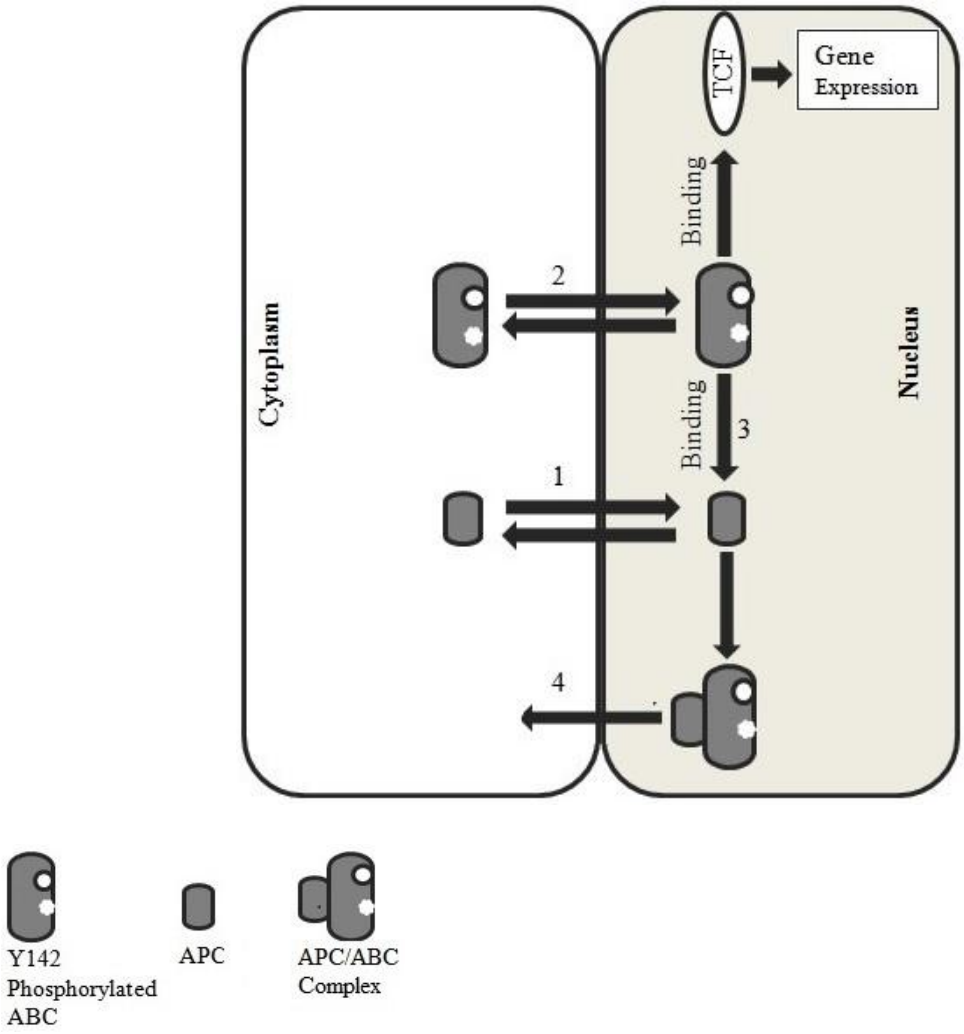


Figure 5.4 Scheme for the possible role of APC in regulating binding of β-catenin to TCF in the nucleus. (1) Shuttling of APC (2) Shuttling of β-catenin (3) APC competing with TCF for binding β-catenin (4) Export of β-catenin out of the nucleus as APC/ABC complex.

5.3.4 Conceptual models of the effects of APC mutation.

Different types of mutations affect the different roles which APC plays in the Wnt/β-catenin signaling pathway differently.

5.3.4.1 APC lacking 20aa and 15aa

APC lacking 20aa sites cannot bind β-catenin in a way that marks it for degradation. We model this by reducing the probability of the transformation of GSK3-PBD to GSK3-PB/ID. Since the 15aa sites of APC can still bind β-catenin, APC lacking only 20aa would still continue to perform its roles of cytoplasmic and nuclear sequestration of β-catenin and its nuclear export. If 15aa sites are also inactive, β-catenin cannot bind to APC at all and APC ceases to have any regulatory role in Wnt signaling.

5.3.4.2 APC lacking SAMP

This is modeled as a reduced probability for Axin to bind to APC. This would result in reduced level of the Destruction Complex because the Destruction Complex forms by the binding of kinases to the APC/Axin complex.

5.3.4.3 APC lacking NLS

This is modeled as reduced probability for APC to translocate to the nucleus.

5.3.4.4 APC lacking NES

This is modeled as reduced probability for translocation of APC and APC/ABC complex from the nucleus to the cytoplasm.

5.4 The computational model.

Following the Chen et al. [399] approach, we have chosen to translate the conceptual model into a stochastic simulation model solved numerically by the Gillespie algorithm [332]. A stochastic simulation model is a better representation than ODEs for a cellular system in which discreteness and stochasticity may play important roles especially in cases where the molecular populations of at least some of the reactant species are not many orders of magnitude larger than one [332].

We consider that the N molecular partners {S[1].....S[N]} of this network of reactions interact through M reactions {R[1], . . . , R[M]}. In this formulation, a different state or a different sub-cellular location of the same molecule is considered to be a different species. Thus, for example, APC in cytoplasm and APC in nucleus are considered to be two different species of molecules.

x[i][t] denotes the number of molecules of the species of S[i] in the system at time t. The state of the system at any point in time is depicted as the state vector X(t) ≡ (x[1][t], . .x[N][t]). The goal of the simulation is to determine X(t) at any point of time assuming an initial state $X[t_0]=X_0$ at some initial time t_0.

Each reaction R[j] is represented by two quantities:

The first is the rate of the reaction k[i] from which the probability of occurrence of the reaction can be calculated. The other its state-change matrix V, where ν[i][j] , is the change in the S[i] molecular population caused by one reaction R[j]. The details of the system viz. set of molecular species, S and the vector, $X[t_0]$ that gives the initial concentrations of all the molecular species are given in Table 5.A.1 of Appendix 5.A. The set of reactions R[j] that the molecules can undergo, along with the reaction constants k[j] and the change in the number of molecules of each species due to the reaction are given in Table 5.A.2 of Appendix 5.A. Most of the values are the same as those used in the Chen et al. [399] model after converting concentrations to number of molecules assuming a sub-cellular system size of 2.5% of a cell volume of 10^{-12} m^3. A few additional rate constants and initial values are introduced to take care of the new reactions in our model. We follow the Gillespie algorithm to determine the evolution of the number of molecules of each species in time as they interact with each other and/or change their state. The details of the numerical method are given in Appendix 5.A**.**

5.5. Results and discussion

The simulations output the time evolution of the number of all the different molecular species considered in our model. All the Tables and Figures presented here, (except Figure 5.6) show the computed time averaged values of the ABC/TCF complex for various values of the model parameters. The time averaging is done over a period of 10 hours starting from time=0 and ending at time=10 hours.

5.5.1 Sensitivity of the results to the newly assumed rate constants.

In our simulations we mostly use the rate constants and initial values used in the Chen et al. [399] model. The introduction of the additional regulatory roles of APC in Wnt signaling has brought in three additional rate constants into the simulation over and above those used by Chen et al. [399] in their simulations. These are:

1. The binding constant of Active β-catenin (ABC) to APC
2. The rate of nuclear translocation of APC
3. The rate of nuclear export of APC/ABC complex.

The importance of the additional regulatory roles of APC (other than through the Destruction Complex) that we have introduced into the model would depend on the strength of the APC/ABC binding constant and the rates of nuclear shuttling. In the absence of experimental values for these parameters, we use the computational model to analyze the sensitivity of the outcome to these reaction rates. All the four additional roles of APC in regulating the nuclear level of β-catenin/TCF depends primarily on the binding of APC to ABC, since only the activated form of β-catenin can translocate to the nucleus [404]. So in our computational experiments, we can effectively cut off the additional regulatory mechanisms by assuming that APC binds only to unphosphorylated β-catenin and not to the phosphorylated forms of β-catenin.

Figure 5.5 shows the sensitivity of the average level of the ABC/TCF complex to the binding affinity level assumed for ABC to APC. The binding affinity level is the ratio of the value we assume for APC/ABC binding to the Chen et al. [399] value for APC/β-catenin. We find that binding affinity ratio of 0 and 1 have the same outcome. Thus assuming that ABC has the same affinity for APC as unphosphorylated inactive β-catenin (binding affinity ratio=1), the additional regulatory roles that we have introduced do not make significant changes in the level of ABC/TCF. Therefore if sequestration of β-catenin by APC is to have any effect on the level of β-catenin/TCF, APC must have a higher affinity for phosphorylated β-catenin (ABC) than unphosphorylated β-catenin. When we assume that the affinity of ABC to APC is 10 times that of unphosphorylated β-catenin, we find that the additional regulatory mechanisms reduce the level of ABC/TCF by a factor of nearly 2. On assuming that the affinity of ABC to APC is 100 times that of unphosphorylated β-catenin, the sequestration of ABC by APC is so strong that even under Wnt "On" conditions, there is very little ABC/TCF formation. In the rest of our results we have used a binding constant of 5.55556E-05 ($Molecules^{-1}Minute^{-1}$) as the "normal" (unmutated) value for ABC binding, which is 10 times the value used for unphosphorylated β-catenin binding.

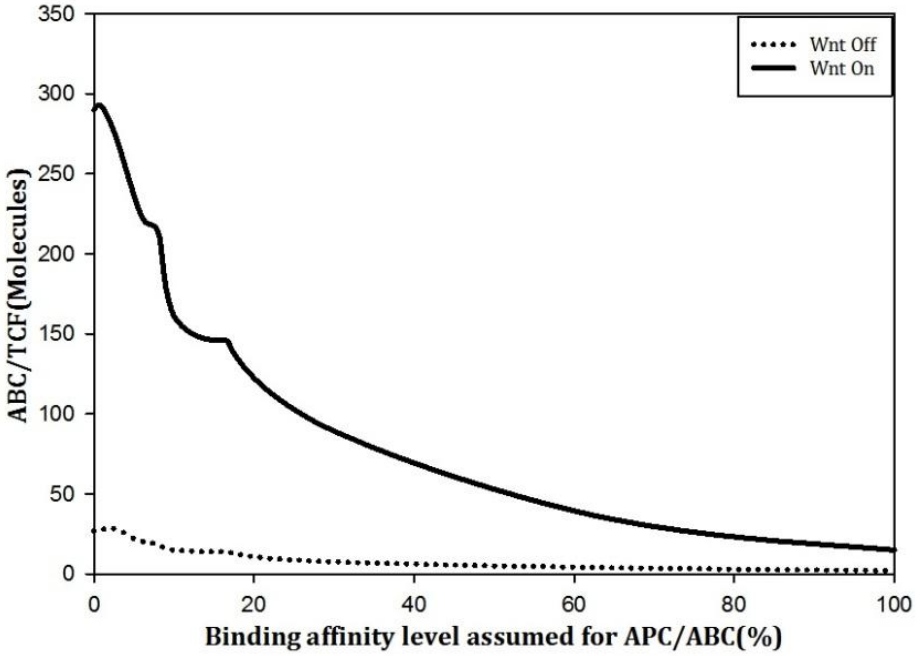


Figure 5.5 Sensitivity of the average (over 10 hours) number of molecules of ABC/TCF to the binding affinity level assumed for APC/ABC. The binding affinity level is expressed as the ratio of the APC/ABC binding constant to the APC/ β-catenin (unphosphorylated) binding constant.

In Table 5.2 and Table 5.3 we show the sensitivity of ABC/TCF level to the rates of nuclear translocation and nuclear export of APC and APC/ABC respectively. The change in the level of ABC/TCF is seen to be marginal under the range of rate constants studied. In the rest of the simulations we use the value 0.33 Minute^{-1} for both the rate constants.

Table 5.2 Sensitivity of ABC/TCF level to the rate constant assumed for APC translocation.

APC Translocation Rate Constant (Minute^{-1})	**ABC/TCF (Molecules) Wnt "Off"**	**ABC/TCF (Molecules) Wnt "On"**
0.00033	19	207
0.0033	17	197
0.033	14	163
0.33	13	157

Table 5.3 Sensitivity of ABC/TCF level to the rate constant assumed for nuclear export of APC/ABC.

APC/ABC Nuclear Export Rate Constant (Minute^{-1})	ABC/TCF (Molecules) Wnt "Off"	ABC/TCF (Molecules) Wnt "On"
0.33	18	184
0.033	23	234
0.0033	21	227
0.00033	23	226

5.5.2 Kinetic response of β-catenins to Wnt stimulation.

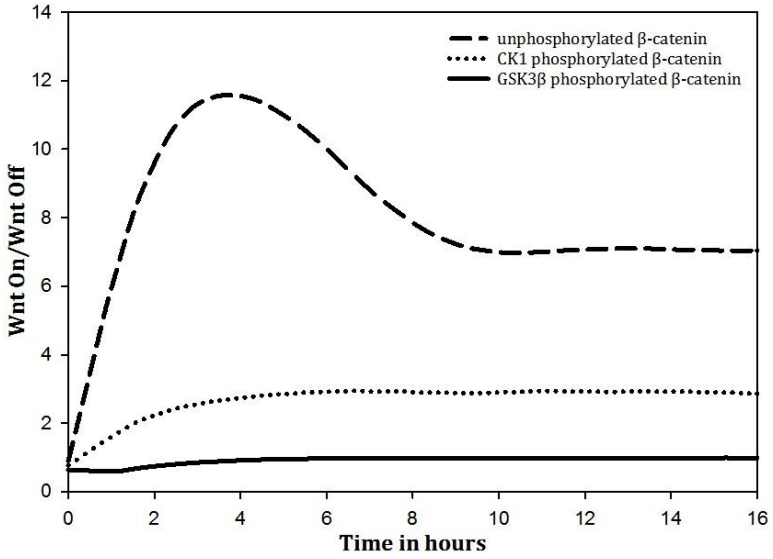


Figure 5.6 Computed kinetic response of unphosphorylated and phosphorylated β-catenins to Wnt stimulation.

Figure 5.6 shows plots of the Wnt "On" to Wnt "Off" ratios of the computed levels of free unphosphorylated β-catenin, CK1 phosphorylated β-catenin and GSK3β phosphorylated β-catenin bound to the Destruction Complex. It is seen that under continuous Wnt "On" conditions, the level of free unphosphorylated β-catenin rises to about 12-fold the Wnt "Off" value, and then falls to a steady value of around 7. The levels of phosphorylated β-catenins bound to the Destruction Complex, fall slightly initially and then become steady with the GSK3β phosphorylated β-catenin resuming the Wnt "Off" level and the CK1 phosphorylated β-catenin becoming 3 times the Wnt "Off" level. These trends are qualitatively similar to experimental observations [312].

5.5.3 Effect of APC mutations.

Since this model explicitly includes the multiple roles played by APC in regulating Wnt signaling, it can be used to simulate the effects of different types of APC mutations on Wnt signaling. Suppression of the regulatory functions of APC becomes significant when there is elevation of ABC/TCF levels even under Wnt "Off" conditions leading to constitutive activation of the Wnt/β-catenin signaling pathway.

5.5.3.1 Effect of inactivation of 20aa sites.

As mentioned in Section 5.3.4.1, the effect of inactivation of 20aa sites on the APC protein is modeled by reducing the probability of Step 1 of the Destruction cycle (Section 5.3.1), viz. the structural transformation of the Destruction Complex. Since the 15aa sites can still bind β-catenin, APC still plays a role in regulating Wnt signaling by the binding and sequestration of ABC. Table 5.4 shows the average number of ABC/TCF complex molecules for different values of the rate constant for the transformation.

Table 5.4 Variation of ABC/TCF with different levels of inactivation of 20aa sites of APC represented as percentage of the "normal" rate constant for transformation of Destruction Complex.

Level of inactivation of 20aa sites	ABC/TCF (Molecules) Wnt "Off"
100% (fully active)	17
20%	35
10%	63
4%	190
2%	541
1%	1080
0%	2037

It can be seen that reducing the value of the transformation rate constant to less than 10% of the "normal" value of 5 (Minute^{-1}) [399] results in a significant increase of ABC/TCF even in the absence of Wnt signals. Complete inactivation of the transformation step leads to steep, continuous increase of the level ABC/TCF complex.

5.5.3.2 Effect of inactivation of both 20aa and 15aa sites.

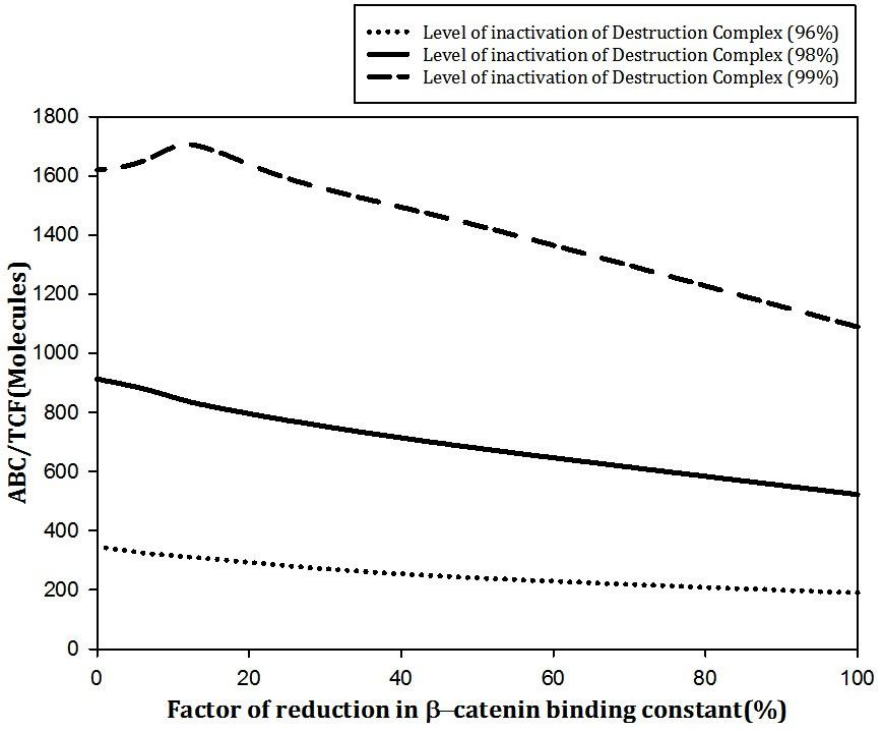


Figure 5.7 Effect of inactivation of both 15aa and 20aa sites under Wnt "Off" conditions. Effect of inactivation of binding sites is depicted on the x-axis as percentage of the "normal" value of the β-catenin binding constant. The three curves show different values of the rate constant for the level of inactivation of Destruction Complex (96%, 98% and 99% of the normal value)

Inactivation of 20aa sites result in reduction of the probability of structural transformation of the Destruction Complex and inactivation of 15aa causes reduction in the binding affinity of β-catenin for APC and consequently lowered sequestration of β-catenin by APC. Figure 5.7 shows that when APC mutations cause lowered sequestration of β-catenin (X-axis), the level of ABC/TCF increases significantly even under Wnt "Off" conditions.

5.5.3.3 When the Destruction Complex formation is affected by SAMP mutation.

SAMP mutations reduce binding of Axin to APC. Since the model assumes that the Destruction Complex is formed by binding of GSK3β to the APC/Axin complex,

reduction in the formation of APC/Axin complex would result in lowered levels of the Destruction Complex.

Table 5.5 Effect of SAMP inactivation. Level of inactivation is depicted as percentage of the "normal" Axin/APC binding constant (0.00011 Molecule^{-1}Minute^{-1}).

Level of inactivation of SAMP	ABC/TCF (Molecules) Wnt "Off"	β-catenin (Molecules) Wnt "Off"	Destruction Complex (Molecules) Wnt "Off"
100%	128	4622	122
10%	186	14831	11
1%	46	28543	1
0.1%	5	32421	0
0.01%	4	32847	0

Table 5.5 gives the ABC/TCF levels along with the cytoplasmic β-catenin levels and number of Destruction Complexes for different values of the Axin/APC binding constant. The initial number of the Destruction Complexes is assumed to be zero in these simulations.

Cytoplasmic β-catenin level increases steadily with decrease of Axin/APC binding constant because of the reduced number of Destruction Complexes. However this β-catenin would remain in the cytoplasm because it is not converted to the active form, ABC in the absence of the CK1 phosphorylation step in the Destruction Complex and only ABC that is capable of translocating to nucleus and binding to TCF. Therefore the ABC/TCF level is seen to decrease when the Axin/APC binding constant goes below 10% of its normal value.

We could hypothesize that there could be other ways in which β-catenin could be phosphorylated to ABC, independent of the Destruction Complex. With this hypothesis and assuming different probabilities for the Destruction-Complex-independent phosphorylation of β-catenin to ABC we get a steady increase in ABC/TCF levels as the Axin/APC binding constant is reduced (Table 5.6).

Table 5.6 ABC/TCF and β-catenin levels for assumed rate constants for Destruction Complex-independent-phosphorylation of β-catenins. SAMP inactivation level is 1% of the wild value.

Rate Constants for Destruction Complex- Independent - Phosphorylation of ABC (Minute^{-1})	ABC/TCF (Molecules) Wnt "Off"
0.000033	46
0.00033	300
0.0033	2103
0.033	5388
0.33	5975

5.5.3.4 Mutation of sites involved in nuclear shuttling.

As already seen in the sensitivity analysis (Table 5.2 and Table 5.3), the changes to nuclear shuttling created by mutations to the NLS or NES sites would make only marginal changes to ABC/TCF levels. We can generalize that mutations that affect nuclear shuttling of APC plays a minor role in comparison to the mutations that affect the cytoplasmic level of active β-catenin.

5.6 Conclusions

A computational model that incorporates five different regulatory roles of APC in the Wnt/β-catenin signaling pathway has been developed. It is possible to use the computational model for performing various kinds of simulation experiments to study the relative effects of these different roles. The experiments we performed indicate that the major regulatory role of APC in Wnt signaling is its role in the degradation of β-catenin. Consequences of different mutations of APC were simulated under Wnt "Off" conditions to identify which mutations are capable of producing activation of the Wnt/β-catenin signaling pathway even in the absence of Wnt signals. The inactivation of the 20aa sites that play the major role in the destruction cycle and the inactivation of SAMP sites that prevent formation of the Destruction Complex are shown to have the biggest effects in creating the constitutive activation of the Wnt/β-catenin signaling pathway. This is in agreement with previous reports that the most frequently occurring

APC mutants in cancer are truncated forms of APC that lack 20aa sites and SAMP sites [98, 393].

We find that cytoplasmic retention by sequestration of β-catenin by APC, can play a significant regulatory role only if the binding affinity of S45-phosphorylated, active β-catenin (ABC) to APC is at least an order of magnitude greater than that of unphosphorylated β-catenin. The roles played by the nuclear sequestration and export of β-catenin by APC does not appear to be significant under the range of parameters studied. Schmitz et al.[398] on the other hand have reported that under certain parameter ranges β-catenin/TCF levels can increase due to nuclear shuttling of APC. In this chapter we have not generated results under changes to the default parameters we inherited from the Chen et al. [399] model and have investigated only the changes due to the new parameters we have introduced in the model. Specifically, Schmitz et al. [398] have found that when they used default parameters from Lee et al. [292] model for the relative dissociation constants for β-catenin binding to APC and TCF, effect of APC shuttling is negligible. A more extensive parametric analysis of our model may therefore address the discrepancy.

5.7 Appendix 5.A - The computational model details

In our model we have a system of M=32 molecular species represented by a Species Vector S. The number of molecules of each species present in the system at any time t is given by the State vector X[t] which can be acted upon and changed by N=70 reactions represented by the Reaction Vector, R. The elements, s[i] of the Species Vector along with the elements, x[i][0] , of the initial State Vector X[0] are given in Table 5.A.1.

Table 5.A.1 The Species Vector with initial State Vector (Abbreviations explained in Table 5.1).

Index - i	Species Vector elements s[i]	State Vector elements x[i][0]
1	Axin/APC	600
2	GSK3	3000
3	D	900
4	BD	60
5	CK1-pBD	30
6	GSK3-pBD	30
7	GSK3-pB/ID	90
8	ID	900
9	ABC_Cyto (ABC in cytoplasm)	30
10	ABC_Nucl (ABC in nucleus)	30
11	WC	0
12	WD	0
13	WBD	0
14	CK1-WBD	0
15	GSK3-WBD	0
16	P_ABC_Cyto (Y142 phosphorylated ABC_Cyto)	30
17	P_ABC_Nucl (Y142 phosphorylated ABC_Nucl)	30
18	ABC/TCF	30
19	Free Cad	0
20	Cad/Cat_Golgi	900
21	Cad/Cat_Cell_Surface	900
22	APC in cytoplasm	3000
23	APC in nucleus	0
24	Axin	300
25	β-catenin in cytoplasm	300
26	APC/β-catenin	3
27	APC/P_ABC_Nucl	0
28	APC/P_ABC_Cyto	3
29	Axin/β-catenin complex	1
30	APC/ABC_Cyto	3
31	APC/ABC_Nucl	0
32	Cad/ABC	0

The elements of the Reaction vector, i.e. all reactions R[j] that can act upon the system, the consequences of each reaction and the respective reaction rate constants k[j] are given in Table 5.A.2.

Table 5.A.2 Reactions, outcome of reactions and rate constants (Abbreviations explained in Table 5.1)

Index - j	Reactions R[j]	Outcome of the Reaction	Rate Constant - k[j]
	Formation and functioning of the Destruction Complex bound to Wnt receptor complexes at the cell membrane		
1	Binding of D to WC D+WC-> WD	D and WC decrease by 1 and WD increases by 1	2.78 $Molecules^{-1}$ $Minute^{-1}$
2	Binding of BD to WC BD + WC -> WBD	BD and WC decrease by 1 and WBD increases by 1	2.78 $Molecules^{-1}$ $Minute^{-1}$
3	Binding of CK1-pBD to WC CK1-pBD + WC -> CK1-WBD	CK1-pBD and WC decrease by 1 and CK1-WBD increases by 1	2.78 $Molecules^{-1}$ $Minute^{-1}$
4	Binding of GSK3-pBD to WC GSK3-pBD + WC -> GSK3-WBD	GSK3-pBD and WC decrease by 1 and GSK3-WBD increases by 1	2.78 $Molecules^{-1}$ $Minute^{-1}$
5	Binding of β-catenin to WD β-catenin + WD -> WBD	WD and β-catenin in cytoplasm decreases by 1 and WBD increases by 1	0.0043 $Molecules^{-1}$ $Minute^{-1}$
6	Phosphorylation of WBD by CK1 WBD->CK1-WBD	WBD decreases by 1 and CK1-WBD increases by 1	4.17 $Minute^{-1}$
7	Dephosphorylation of CK1-WBD CK1-WBD-> WBD	CK1-WBD decreases by 1 and WBD increases by 1	66.67 $Minute^{-1}$
8	Phosphorylation of CK1-WBD by GSK3β CK1-WBD -> GSK3-WBD	CK1-WBD decreases by 1 and GSK3-WBD increases by 1	2 $Minute^{-1}$
9	Dephosphorylation of GSK3-WBD GSK3-WBD-> CK1-WBD	CK1-WBD increases by 1 and GSK3-WBD decreases by 1	1 $Minute^{-1}$
10	Dissociation of WBD WBD->WD + β-catenin	WD and β-catenin in cytoplasm increase by 1 and WBD decreases by 1	16.67 $Minute^{-1}$
11	Transformation of WC- bound Destruction Complex GSK3-WBD-> GSK3-pB/ID+ WC	GSK3-pB/ID and WC increase by 1 and GSK3-WBD decreases by 1	5 $Minute^{-1}$
12	Binding of ABC to WD ABC + WD -> CK1-WBD	ABC in Cytoplasm and WD decrease by 1 and CK1-WBD increases by 1	0.06 $Molecules^{-1}$ $Minute^{-1}$
13	Release of ABC from WD CK1-WBD ->ABC + WD	ABC in Cytoplasm and WD increase by 1 and CK1-WBD decreases by 1	432 $Minute^{-1}$

	Formation and functioning of the Destruction Complex in the cytoplasm		
14	Formation of Destruction Complex Axin/APC + GSK3 -> D	Axin/APC and GSK3 decrease by 1 and D increases by 1	0.00083 $Molecules^{-1}$ $Minute^{-1}$
15	Dissociation of Destruction Complex D ->Axin/APC + GSK3	Axin/APC and GSK3 increase by 1 and D decreases by 1	1.67 $Minute^{-1}$
16	Recovery of Destruction Complex ID -> D	ID decreases by 1 and D increases by 1	0.167 $Minute^{-1}$
17	Binding β-catenin by Destruction Complex D + β-catenin -> BD	D and β-catenin in cytoplasm decrease by 1 and BD increases by 1	0.0043 $Molecules^{-1}$ $Minute^{-1}$
18	Dissociation of BD BD -> D + β-catenin	D and β-catenin in cytoplasm increase by 1 and BD decreases by 1	16.67 $Minute^{-1}$
19	Phosphorylation of BD by CK1 BD ->CK1-pBD	BD decreases by 1 and CK1-pBD increases by 1	4.17 $Minute^{-1}$
20	Dephosphorylation of CK1-pBD CK1-pBD -> BD	BD increases by 1 and CK1-pBD decreases by 1	3.33 $Minute^{-1}$
21	Phosphorylation of CK1-pBD by GSK3β CK1-pBD -> GSK3-pBD	CK1-pBD decreases by 1 and GSK3-pBD increases by 1	6.67 $Minute^{-1}$
22	Dephosphorylation of GSK3-pBD GSK3-pBD -> CK1-pBD	CK1-pBD increases by 1 and GSK3-pBD decreases by 1	1.67 $Minute^{-1}$
23	Transformation of Destruction Complex GSK3-pBD -> GSK3-pB/ID	GSK3-pB/ID increases by 1 and GSK3-pBD decreases by 1	5 $Minute^{-1}$
24	Binding of ABC to D ABC + D -> CK1-pBD	D and ABC in Cytoplasm decrease by 1 and CK1-pBD increases by 1	0.0006 $Molecules^{-1}$ $Minute^{-1}$
25	Release of ABC from D CK1-pBD ->ABC + D	ABC in Cytoplasm and D increase by 1 and CK1-pBD decreases by 1	0.54 $Minute^{-1}$
	Reactions in the cytoplasm		
26	β-catenin retention caused by Axin Axin+ β-catenin ->Axin/β-catenin	Axin and β-catenin in cytoplasm decrease by 1 and Axin/β-catenin complex increases by 1	1.67E-05 $Molecules^{-1}$ $Minute^{-1}$
27	Dissociation of Axin/ β-catenin Axin/β-catenin ->Axin+ β-catenin	Axin and β-catenin in cytoplasm increase by 1 and Axin/β-catenin complex decreases by 1	1.67 $Minute^{-1}$
28	β-catenin retention caused by APC in Cytoplasm APC + β-catenin -> APC/β-catenin	APC in cytoplasm and β-catenin in cytoplasm decrease by 1 and APC/β-catenin increases by 1	5.56E-06 $Molecules^{-1}$ $Minute^{-1}$
29	P_ABC retention caused by APC in Cytoplasm	P_ABC_Cyto and APC in cytoplasm decrease by 1 and	5.56E-05 $Molecules^{-1}$ $Minute^{-1}$

	P_ABC_Cyto+ APC -> APC/P_ABC_Cyto	APC/P_ABC_Cyto increases by 1	
30	ABC retention caused by APC in Cytoplasm ABC_Cyto+ APC -> APC/ABC_Cyto	ABC_Cyto and APC in cytoplasm decreases by 1 and APC/ABC_Cyto increases by 1	5.56E-05 $Molecules^{-1}$ $Minute^{-1}$
31	Dissociation of APC/ β-catenin APC/β-catenin -> APC + β-catenin	APC in cytoplasm and β-catenin in cytoplasm increase by 1 and APC/β-catenin decreases by 1	1.67 $Minute^{-1}$
32	Dissociation of APC/P_ABC_Cyto APC/P_ABC_Cyto -> APC + ABC_Cyto	APC in cytoplasm and ABC_Cyto increase by 1 and APC/P_ABC_Cyto decreases by 1	1.67 $Minute^{-1}$
33	Dissociation of APC/ABC_Cyto APC/ABC_Cyto ->ABC_Cyto+ APC	ABC_Cyto and APC in cytoplasm increases by 1 and APC/ABC_Cyto decreases by 1	1.67 $Minute^{-1}$
34	Y142 Phosphorylation of ABC_Cyto ABC_Cyto ->P_ABC_Cyto	ABC_Cyto decreases by 1 and P_ABC_Cyto increases by 1	0.33 $Minute^{-1}$
35	Dephosphorylation of P_ABC_Cyto P_ABC_Cyto ->ABC_Cyto	P_ABC_Cyto decreases by 1 and ABC_Cyto increases by 1	0.33 $Minute^{-1}$
36	Formation of Axin/APC Axin+ APC ->Axin/APC	APC in cytoplasm and Axin decrease by 1 and Axin/APC increases by 1	0.00011 $Molecules^{-1}$ $Minute^{-1}$
37	Dissociation of Axin/APC Axin/APC ->Axin+ APC	APC in cytoplasm and Axin increase by 1 and Axin/APC decreases by 1	0.17 $Minute^{-1}$
	Reactions in the nucleus		
38	P_ABC retention caused by APC in Nucleus P_ABC_Nucl+ APC -> APC/P_ABC_Nucl	P_ABC_Nucl and APC in nucleus decrease by 1 and APC/P_ABC_Nucl increases by 1	5.56E-05 $Molecules^{-1}$ $Minute^{-1}$
39	ABC retention caused by APC in nucleus ABC_Nucl+ APC -> APC/ABC_Nucl	ABC_Nucl and APC in nucleus decreases by 1 and APC/ABC_Nucl increases by 1	5.56E-05 $Molecules^{-1}$ $Minute^{-1}$
40	Dissociation of APC/P_ABC_Nucl APC/P_ABC_Nucl ->ABC_Nucl+ APC	ABC_Nucl and APC in nucleus increase by 1 and APC/P_ABC_Nucl decreases by 1	1.67 $Minute^{-1}$
41	Dissociation of APC/ABC_Nucl APC/ABC_Nucl ->ABC_Nucl + APC	ABC_Nucl and APC in nucleus increases by 1 and APC/ABC_Nucl decreases by 1	1.67 $Minute^{-1}$
42	Y142 Phosphorylation of ABC_Nucl ABC_Nucl ->P_ABC_Nucl	ABC_Nucl decreases by 1 and P_ABC_Nucl increases by 1	0.33 $Minute^{-1}$
43	Dephosphorylation of P_ABC_Nucl ->ABC_Nucl	P_ABC_Nucl decreases by 1 and ABC_Nucl increases by 1	0.33 $Minute^{-1}$
44	Transformation of P_ABC_Nucl to ABC/TCF P_ABC_Nucl -> ABC/TCF	P_ABC_Nucl decreases by 1 and ABC/TCF increases by 1	0.33 $Minute^{-1}$
45	Transformation of ABC/TCF to	ABC/TCF decreases by 1 and	0.33 $Minute^{-1}$

	P_ABC_Nucl ABC/TCF ->P_ABC_Nucl	P_ABC_Nucl increases by 1	
	Reactions involving E-cadherin		
46	Formation of Cad/Cat complex Free Cad + β-catenin -> Cad/Cat_Cell_Surface	Free Cad and β-catenin in cytoplasm decrease by 1 and Cad/Cat_Cell_Surface increases by 1	0.033 $Molecules^{-1}$ $Minute^{-1}$
47	Dissociation of Cad/Cat complex Cad/Cat_Cell_Surface -> Free Cad + β-catenin	Free Cad and β-catenin in cytoplasm increases by 1 and Cad/Cat_Cell_Surface increases by 1	1.67 $Minute^{-1}$
48	Endocytosis of Cad/Cat complex on cell surfaces	Cad/Cat_Cell_Surface decreases by 1	0.0083 $Minute^{-1}$
49	Endocytosis of Cadherin on cell surfaces	Free Cad decreases by 1	0.0083 $Minute^{-1}$
50	Formation of Cad/ABC complex ABC_Cyto + Free Cad -> Cad/ABC	ABC_Cyto and Free Cad decreases by 1 and Cad/ABC increases by 1	0.033 $Molecules^{-1}$ $Minute^{-1}$
51	Dissociation of Cad/ABC complex Cad/ABC ->ABC_Cyto+ Free Cad	ABC_Cyto and Free Cad increases by 1 and Cad/ABC decreases by 1	1.67 $Minute^{-1}$
	Synthesis and degradation		
52	Synthesis of WC	WC increases by 1	86.5 $MoleculesMinute^{-1}$
53	Synthesis of Axin	Axin increases by 1	5 $MoleculesMinute^{-1}$
54	Synthesis of APC	APC in cytoplasm increases by 1	50 $MoleculesMinute^{-1}$
55	Synthesis of β-catenin	β-catenin in cytoplasm increases by 1	150.5 $MoleculesMinute^{-1}$
56	Synthesis of Cad/Cat in golgi	Cad/Cat_Golgi increases by 1	51.5 $MoleculesMinute^{-1}$
57	Degradation of WC	WC decreases by 1	0.017 $Minute^{-1}$
58	Degradation of Axin	Axin decreases by 1	0.017 $Minute^{-1}$
59	Degradation of APC	APC in cytoplasm decreases by 1	0.017 $Minute^{-1}$
60	Degradation of β-catenin	β-catenin in cytoplasm decreases by 1	0.0017 $Minute^{-1}$
61	Degradation of Cad/Cat golgi	Cad/Cat_Golgi decreases by 1	0.017 $Minute^{-1}$
62	Degradation of ABC_Cyto	ABC_Cyto decreases by 1	0.0017 $Minute^{-1}$
63	Degradation of GSK3-pB/ID GSK3-pB/ID -> ID	GSK3-pB/ID decreases by 1 and ID increases by 1	1.67 $Minute^{-1}$
	Translocation reactions		
64	Translocation of P_ABC_Cyto to nucleus	P_ABC_Cyto decreases by 1 and P_ABC_Nucl increases by 1	0.33 $Minute^{-1}$
65	Translocation of P_ABC_Nucl to cytoplasm	P_ABC_Nucl decreases by 1 and P_ABC_Cyto increases by 1	0.33 $Minute^{-1}$

66	Shuttling Cad/Cat complex from Golgi to cell surfaces	Cad/Cat_Golgi decreases by 1 and Cad/Cat_Cell_Surface increases by 1	0.0405 $Minute^{-1}$
67	Translocation of APC_cyto to nucleus	APC in cytoplasm decreases by 1 and APC in nucleus increases by 1	0.33 $Minute^{-1}$
68	Translocation of APC_nucl to cytoplasm	APC in nucleus decreases by 1 and APC in cytoplasm increases by 1	0.33 $Minute^{-1}$
69	Translocation of APC/ABC from nucleus to cytoplasm	APC/ABC_Nucl decreases by 1 and APC/ABC_Cyto increases by 1	0.33 $Minute^{-1}$
70	Translocation of APC/P_ABC from nucleus to cytoplasm	APC/P_ABC_Nucl decreases by 1 and APC/P_ABC_Cyto increases by 1	0.33 $Minute^{-1}$

From Tables 5.A.1 and 5.A.2, we can determine the elements v[i][j] of the State Change Matrix V . V[i][j] depicts the change in the number of molecules of species [i] that occurs when the reaction R[j] occurs in the system.

For example, let us consider the first reaction R[1] from Table 5.A.2

The reaction R[1] is:

$$WC + D \longrightarrow WD$$

i.e The Destruction Complex D binds to the Wnt receptor complex WC and forms the complex WD. Thus, the consequence of reaction R[1] is that, the number of WC and D reduce by one and the number of WD species increases by one. Since as per Table 5.A.1, WC is the 11^{th}, element S[11] , D is the 3^{rd} element S[3] and WD is the 12^{th} element S[12] of the Species Vector, we can write:

$$v[11][1] = -1$$
$$v[3][1] = -1$$
$$v[12][1]=1$$
$$v[i][1] = 0 \text{ for all values of i not equal to 3, 11 or 12}$$

The probability per unit time that the reaction R[j] occurs, called the Propensity Function a[j] for each reaction at any time t can be calculated from the rate constant k[j] of the reaction and the State Vector at that time, X[t].

For example, the Propensity Function of the reaction R[1],

$$a[1] = k[1] * x[3]*x[11]$$

In the Stochastic Simulation Algorithm, at any time t we first determine the time interval in which the next reaction in the system will take place as:

$$\tau=1/a_0*\log(1/u_1) \quad (1)$$

where u_1 is a random number between 0 and 1 and

$$a_0= \sum_{j'=M}^{j'=1} a[j'] \quad (2)$$

The reaction index "jnext" of the reaction that will occur in this time interval is calculated as:

jnext is the smallest integer satisfying

$$\sum_{j'=jnext}^{j'=1} a[j'] > u_2 * a_0 \quad (3)$$

where u_2 is a second random number between 0 and 1

The algorithm is as follows:

0 Initialize the time t =0 and the system's state X=X[0].

1. With the system in state X[t] at time *t*, evaluate all the a[j]s and their sum a_0
2. Generate two random numbers u_1 and u_2 and generate values for τ and jnext using equations (1) and (3)
3. Calculate the elements of the State Vector X[t+τ] as :

$$x[i][t+\tau] = x[i][t]+v[i][jnext] \quad (4)$$

3. Replace $t \leftarrow t + \tau$ and X[t]← X[t+τ]
4. Return to Step 1

6

Extension of the Crypt Model - Multiple Ways in which APC Mutations Initiate Colon Cancer

In Chapter 3 we developed an Agent-based model for simulating the dynamic processes that maintain homeostasis in the colon crypt. In this chapter we extend the Agent-based model of the colon crypt to include explicitly the multiple roles played by the APC protein in maintaining the fidelity of these processes. We use this model to simulate the changes that would occur when the APC functions are perturbed by mutations in the gene. The overall consequences of perturbations to the different processes are observed and understood through controlled simulation experiments.

6.1 Biological background - Cell functions affected by APC mutations

APC has been described as a "scaffold that has its finger in every epithelial biology pie" [406]. APC is known to contribute to the fidelity of all the dynamic processes, such as division, contact inhibition, migration, differentiation and death that maintain the integrity of the colon crypt [407]. APC mutation is in most cases the first mutation that appears in colon tumors. Deletion of APC within the intestinal crypt stem cells leads to adenoma formation in as little as 3 weeks, suggesting that APC loss alone is sufficient for benign tumor growth [9].However, for development of full-fledged adenocarcinoma, additional mutations are required in the form of either loss of function mutations in other tumor suppressors-e.g., PTEN-or gain of function mutations of oncogenes, such as K-ras [408, 409]. The fact that APC mutation is the initial mutation implies that the initial APC mutation also facilitates the acquisition of additional mutations by the cell.

6.1.1 APC role in cell division driven by Wnt signaling

The most well established function of APC in the colon crypt is its role as a negative regulator of Wnt signaling [283]. APC, by its participation in the destruction of β-catenin (dealt with in detail in Chapter 5) prevents the entry of the cell into cell cycle. A

decreasing concentration gradient of Wnt signaling factors in the surroundings, along with an increasing expression of APC within the cell, regulates cell proliferation and promotes cell differentiation along the crypt axis [152]. Mutation of APC perturbs the regulatory function of APC and allows the Wnt/β-catenin signaling pathway to be activated even in the absence of Wnt signals.

6.1.2 APC role in contact inhibition of proliferation and migration

Contact inhibition is a key anticancer mechanism that arrests cell cycle or proliferation when cells reach a high density. Contact inhibition refers to two distinct but closely related phenomena: Contact Inhibition of Locomotion (CIL) and Contact Inhibition of Proliferation (CIP). Loss of APC has been reported to cause defects in cell migration and cell adhesion due to the stabilizing effects of APC on cytoskeletal proteins, including F-actin and microtubules [188]. The regular clearance of cells from the epithelium by migration and shedding ensures that cells do not remain long enough in the crypt to acquire and stabilize transforming mutations. Therefore defects in migration caused by APC loss, that increase the retention time of cells in the highly proliferative environment of the crypt, can set the stage for acquisition of additional mutations that cooperate to drive tumor progression into carcinoma.

6.1.3 APC role in serial differentiation

In APC mutated crypts, the proliferative hierarchy of arrangement of cells in the crypt, with the proliferative potential decreasing progressively from the base, is lost. Intestinal cells lacking APC are highly proliferative and fail to differentiate [410]. It has been observed that APC deficiency also leads to the production of daughter cells that have many properties of premalignant cells, including both failed differentiation and increased proliferation [49].

6.1.4 APC role in cell shedding

Apoptotic rates are highest at the top of the crypt [411]. A key mechanism of apoptosis within the normal homeostatic epithelium is anoikis [412] , whereby loss of contact with the extracellular matrix induces apoptosis. This is known to occur in colonic crypts and its dysregulation is thought to play a role in CRC progression and crypt homeostasis

[262]. APC role in cell adhesion (Section 1.4.3.2 of Chapter 1) can play a part in the phenomenon of cell shedding and anoikis.

6.1.5 APC role in stem cell division

As described in Chapters 3 and 4, stem cells have two modes of division, symmetric and asymmetric. Asymmetric division ensures production of Transit Amplifying cells that come out of the stem cell compartment while symmetric division balances accidents that disturb the homeostasis of the stem cell compartment. The balance between the two modes of stem cell division has been found to be defective in disease states like cancer [366]. Anchoring of stem cells in the niche appears to play an important role in the decision to divide symmetrically or asymmetrically [15, 365, 374] as well as the decision to differentiate or not [375, 376]. When APC involvement in the stabilization of cytoskeletal proteins is perturbed by its mutation, the balances that maintain homeostasis can be affected. In Chapter 4 we proposed a hypothesis on the manner in which APC mutations can disrupt this balance and initiate colon cancer. We showed that the hypothesis , that APC mutations increase the level of symmetric division and bias it towards production of stem cell progeny, is able to explain several of the changes that are observed in the initial stages of transformation [363].

6.2 Modeling the perturbations in the colon crypt due to APC mutation

We extend the Agent-based crypt model described in Chapter 3 to explicitly represent the multiple functions of APC in the processes of Wnt/β-catenin signaling pathway driven division, contact inhibition, migration, serial differentiation, and stem cell division. For this we add a few more attributes to the cell agents over and above those described in Chapter 3. We also introduce new APC dependent variables to represent the role of APC in the cell processes (Agent actions) that govern homeostasis in the crypt, so that the perturbations to these processes by APC mutation can be modeled.

6.2.1 Modeling perturbations to cell division by Wnt signaling

In the model of crypt described in Chapter 3, we had explicitly included APC in the cell division process. The most well known role of APC is the regulation of the Wnt/β-catenin signaling pathway. As detailed in the molecular level model of Chapter 5, APC regulates the Wnt/β-catenin signaling pathway in multiple ways. Wnt/β-catenin

signaling pathway essentially signals the cell to enter the cell cycle and APC is a repressor of this signal. Therefore in our tissue level model APC regulation of the Wnt/β-catenin signaling pathway was modeled as:

$$P_{wnt} = (1+Wnt)(1-0.5*APC_{conc}) \quad (6.1)$$

Where P_{wnt} is the probability of the transition from the quiescent state to the cell cycle. Wnt is the concentration of Wnt signaling molecules in the environment of the cell and APC_{conc} is the concentration of APC expressed within the cell. Wnt concentration is assumed to vary from 0 to 1 having maximum value at the stem cell compartment and decreasing linearly along the crypt axis and falling to zero at about half way up the crypt.

$$Wnt\ (y) = 1- (y-y_0)/(y_d - y_0) \quad (6.2)$$

Where y denotes the position of the cell along the crypt axis and y_0 and y_d are the positions of the stem cell niche and start of Wnt-free zone respectively.

The level of APC expressed within a cell is assumed to start from a minimum value of 1 in stem cells and increase as the cell gets more and more differentiated, reaching a value of 2 in fully differentiated cells. The level of differentiation of a cell is determined by an attribute Stemness. Stem cells have a Stemness value of 1 and each successive division brings down the Stemness value and it becomes zero (fully differentiated in a certain number of divisions, specified by the parameter Num_div_max (see Chapter 3). Decrease of Stemness per division is therefore equal to 1/Num_div_max. Expression of APC in a cell is related to the degree of differentiation of the cell. Stem cells express the least amount of APC (assumed as 1) and fully differentiated cells express the maximum amount of APC (assumed as 2)

APC concentration within a cell is determined as

$$APC_{conc} = 2 - Stemness \quad (6.3)$$

Equation (6.1) and Eqn. (6.3) together imply that the fully differentiated cells (Stemness=0) cannot enter into cell cycle because the probability of entering cell cycle becomes zero.

We implement APC mutation in a slightly different way from the original model of Chapter 4. In Chapter 4 we used a binary attribute called “Mutation State” to represent

the mutation state of the cell agent. Here we use an attribute "Mutation" that can take any value from 0 to 1 denoting the level of functional APC expressed in the cell. A mutation value of zero represents no mutation and 1 represents complete absence of functional APC protein in the cell.

Mutated APC_{conc} in a cell is given by:

$$APC_{conc} = \text{Normal } APC_{conc} (1\text{-Mutation}) \quad (6.4)$$

where Normal APC_{conc} is given by Equation 6.3.

Equation (6.4) along with Equation (6.1) imply that, as mutations decrease the level of functional APC in the cell, the probability for the cell to shift from a state of quiescence into cell cycle increases and the cell becomes more proliferative.

One of the consequences of APC mutation is that the cell can enter the cell cycle even when there is no Wnt signal, i.e. APC mutation can cause constitutive activation of the Wnt/β-catenin signaling pathway [Chapter 5]. Equation (6.1) allows a finite probability for the cell to enter cell cycle even in the Wnt-free regions of the crypt beyond y_d for mutated cells that may have APC_{conc} less than 2 thereby simulating constitutive activation of the Wnt/β-catenin signaling pathway.

6.2.2 Modeling perturbations to contact inhibition of proliferation

We introduce a variable "Contact_Inhibition" and assume that the probability of entering cell cycle is inversely dependent on contact inhibition

$$P = P_{wnt}/\text{Contact\_Inhibition} \quad (6.5)$$

Contact_Inhibition is taken as proportional to the local cell density which is the number of cells occupying the same grid location (Occupancy of the Grid).

$$\text{Contact\_Inhibition} = K*\text{Occupancy of the grid} \quad (6.6)$$

This ensures that as the grid gets crowded contact inhibition increases.

The proportionality constant K is assumed to be related to APC_{conc} as:

$$K= APC_{conc}/2 \quad (6.7)$$

Equation (6.7) implies that cells expressing lower levels of APC are more tolerant of cell crowding as far as inhibition of cell division is concerned. Thus as APC gets mutated,

contact inhibition decreases and APC mutation reduces the effect of contact inhibition on the probability of transition from Quiescent to G1 state.

6.2.3 Modeling perturbations to migration

In Chapter 3 the rule for migration was developed as:

"The cells are assumed to move up by mitotic pressure. When new cells are born at the bottom, older cells get pushed up due to mitotic pressure. The model implements this through the rule that whenever more than one cell occupies the same grid space, the oldest cells are made to move up by one grid space. However stem cells never move out of the niche."

When cells divide and crowd at a grid location they get compressed and get pushed up. In the original model crowding is defined as "more than one cell occupying the same grid space".

In the new model we introduce a new attribute "Maximum Occupancy" for the cell agent. Since the mechanical force that pushes a cell out of a crowded grid space is generated by the mechanical properties of the cell, the number of cells that can be accommodated in a grid space is a function of the mechanical properties of the cells that occupy the space. The “Maximum Occupancy” attribute for a normal cell is assumed to be 1 so that whenever more than one cell occupies the same grid space, one of them is pushed up. We also assume that it is the more differentiated cell that gets pushed up, This ensures that the upper region of the crypt is more differentiated than the lower.

APC mutation is assumed to increase the Maximum Occupancy of a cell. This implies that more mutated cells can occupy the same location before sufficient mechanical force is generated to push one of the cells up.

$$\text{Maximum Occupancy} = \text{INT}(2/\langle APC_{conc} \rangle) \tag{6.8}$$

Where $\langle APC_{conc} \rangle$ is the average APC_{conc} of the cells in the grid and INT denotes that only the integer part of $2/\langle APC_{conc} \rangle$ is taken as “Maximum Occupancy”

When mutations reduce the concentration of functional APC, more cells are able to crowd into a grid space without pushing up a cell. Therefore migration gets slower in APC mutated cells.

6.2.4 Modeling perturbations to differentiation

Every division of the Transit Amplifying cells gives rise to 2 daughter cells that are more differentiated than the mother cell. We represent the level of differentiation by the attribute, "Stemness" of the cell. Therefore every division produces daughters with lower "Stemness", the decrease in "Stemness" being equal to 1/Num_div_max where Num_div_max is a parameter that specifies the maximum number of divisions a cell can make until it is fully differentiated.

In this model we assume that in APC mutated cells, the mutation changes the "Stemness" that occurs on every division as:

"Stemness" of daughter cell="Stemness" of mother cell-(1-Mutation)/Num_div_max (6.9)

6.2.5 Modeling perturbations to cell shedding

In the original version of the model (Chapter 3) the cell agents that reach the top boundary are removed from the system to simulate shedding of the cell at the top of the crypt. In the new version we make the shedding process dependent on APC concentration by defining a probability of shedding

$$P\_shedding = APC_{conc}/2 \quad (6.10)$$

As APC_{conc} reduces due to mutation, the probability of shedding becomes smaller and mutated cells tend to get retained at the crypt boundary.

6.2.6 Modeling perturbations to stem cell division

Here we implement in the new model, our hypothesis (Chapter 4) that APC mutations increase the probability of symmetric stem cell division, and biases symmetric division towards self-renewal. We relate the Symmetric division probability (P_s) and Differentiation probability (P_d) to APC Mutation as:

$$P_s = \text{Normal } P_s*(1+\text{Mutation}) \quad (6.11)$$

and

$$P_d = \text{Normal } P_d*(1-\text{Mutation}) \quad (6.12)$$

Normal P_s is input as a parameter and Normal P_d is calculated by Eqn. (4.1) of Chapter 4. Viz.

$$P_d = 0.5 - (1-N_S/N_0) \quad (6.13)$$

The summary of the different models is given in Table 6.1

Table 6.1 Summary of the models for the phenomena affected by APC mutation in the crypt

Phenomenon	Model
Mutation of APC	Normal APC_{conc} = 2- Stemness Mutated APC_{conc} = Normal APC_{conc} *(1-Mutation)
Division by Wnt signaling	Probability of entering cell cycle, P_{wnt}= (1+Wnt) *(1- APC_{conc} /2)
Contact Inhibition	Contact_Inhibition = 2*Number of cells in grid/ Normal APC_{conc} allowed in grid Probability of entering cell cycle, $P_{=}$ P_{wnt} /Contact_Inhibition
Migration	Maximum Occupancy = 2/<APC_{conc}> Cells are pushed up when Occupancy of a grid>Maximum Occupancy
Differentiation	Change in Stemness on division – (1-Mutation)/Num_div_max
Shedding	Probability of shedding= APC_{conc}/2
Stem cell division	P_s = Normal P_s* (1+Mutation) and P_d= Normal P_d*(1-Mutation)

6.3 Results and discussion

6.3.1 Perturbing the crypt processes in isolation

The model is used as a virtual experimental apparatus with which we can study how the different crypt processes, perturbed by APC mutation affect the overall crypt homeostasis. Unlike in real experiments in which several processes are likely to be perturbed together by the same APC mutation, in virtual experiments it is possible to apply the perturbations to the processes in isolation or in defined combinations. This can lead to better understanding of the contribution of each perturbation to the overall changes in the crypt and how the perturbations affect each other when they occur together.

As summarized in Table 6.1, we have different variables that are used to model how a cell agent participates in the six different processes (Wnt-signal-driven cell division, migration, contact inhibition, differentiation, cell shedding and stem cell division) that maintain the crypt. All these variables can be perturbed by APC mutations in the cell. However we can isolate the perturbation to a single process by using the normal APC concentration in all the processes except the one we want to study in isolation.

We use 5 indicators to reflect the overall changes brought by the APC mutation:

1. Retention time in the crypt
2. Extent of the proliferative zone
3. Percentage of proliferating cells
4. Total number of cells
5. Survival Advantage to mutated cells

At time=0 a mutation of specified value is introduced into one of the ancestral stem cells in the stem cell niche. The mutation is allowed to disturb only one of the 6 crypt processes at a time.

The parameter values used are given in Table 6.2. We use a smaller representation of the crypt (10 x 20) and also depict time in iteration steps instead of real time. It must be noted that the results presented here are intended to show trends of behavior rather than quantitative values.

Table 6.2 Parameter values

Parameter	Value
Number of columns	10
Number of rows	20
Initial number of stem cells	10
Initial number of cells	200
Cell Cycle Time for Stem cells	48 iterations
Cell Cycle Time for Transit Amplifying cells	24 iterations
Time in G1 state by Stem cells	24 iterations
Time in G1 state by Transit Amplifying cells	8 iterations
The maximum number of divisions before terminal differentiation – Num_div_max	5
Probability of Symmetric Division	0.1
Mutation	0.9

The results of the isolated perturbation experiments are summarized in Table 6.3

Table 6.3 Changes in the crypt due to perturbations of single processes (One stem cell is mutated is mutated so as to express only 10% functional APC)

Different Perturbations	***Retention Time (iteration steps)***	***Proliferative Zone (% of crypt occupied by proliferating cells)***	***Proliferating Cells (% of total number)***	***Survival Advantage to the Mutated Progeny***	***Number of Cells***
No Perturbation	65 - 75	25%	28%	None	224 - steady
Wnt-driven Division	65-75	30%	40%	None	229 - steady
Contact Inhibition	65-75	35%	30%	None	222- steady
Migration	430-450	15% - 20%	20%	Yes - other lineages survivive in small numbers	Increas-ing with time
Cell Shedding	65-75	25%	29%	None	225 - steady
Differentiation	65-75	Extends to the top	53%	Yes - other lineages survivive in small numbers	227 - steady
Stem Cell Division	65-75	Extends to the top	56%	Yes - mutated lineage takes over the crypt completely	Increas-ing with time

Retention time is increased significantly by the perturbation of migration by APC mutation. With the weakening of the cytoskeleton postulated as a consequence of APC mutation, more cells need to crowd in a grid location before sufficient force is built up to push up the cells. It is seen that when the migratory function of the mutated cells is

perturbed there are more cells in the crypt due to increased retention time of the mutated cells. However, unexpectedly, the percentage of cells in the proliferating pool is lower than under the unperturbed condition and the extent of the proliferative zone is also reduced.

Figure 6.1 shows the number of cells from the 10 stem cell lineages as a function of time under the condition of perturbed migration. The stem cell PC4 harbors a 90% APC mutation that is allowed to influence only the migratory function of its progeny. It can be seen that the mutation gives the progeny of PC4 a clear survival advantage over the other lineages.

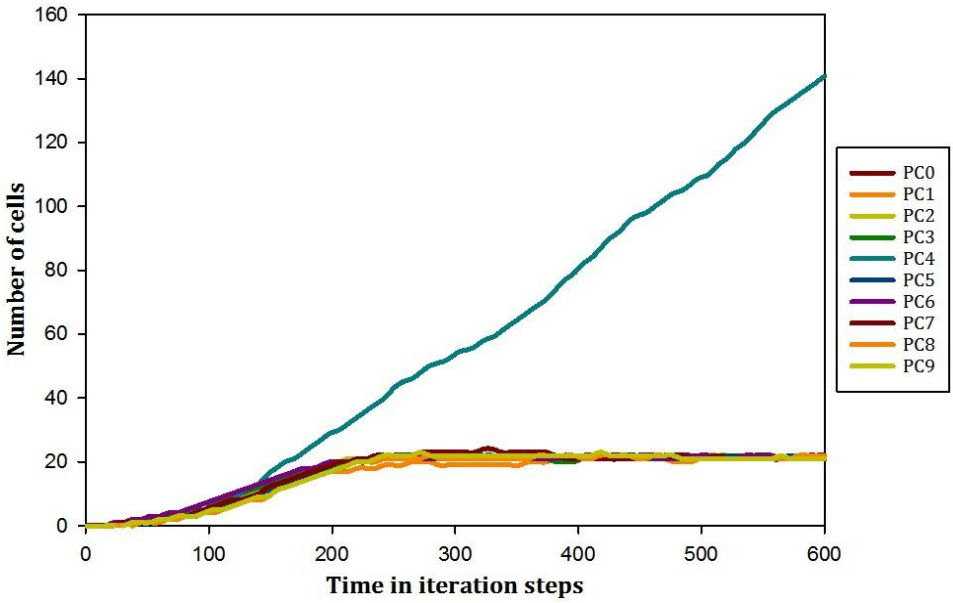


Figure 6.1 Number of cells from the 10 ancestral stem cell lineages as a function of time. Stem cell PC4 and its progeny (blue line) has an APC mutation that affects its migration function alone.

Perturbations to proliferation, differentiation and stem cell division functions result in larger proliferation pool and extended proliferation zone.

However the mutated cell lineage does not get any survival advantage on perturbing the Wnt-driven division process alone. This is because migration responds to increased mitotic pressure by pushing out the mutated cells faster.

When differentiation or stem cell division is perturbed the mutated cell lineage gets survival advantage. The effects are most dramatic for perturbation of stem cell division. The otherwise random process of niche succession (Chapter 3 and 4) gets biased in favor of the mutated stem cell progeny and the mutated cell lineage completely takes

over the stem cell niche and the crypt (Figure 6.2). In the case of perturbations to migration (Figure 6.1) and differentiation, the number of mutated cells increases, but the other lineages also survive in small numbers.

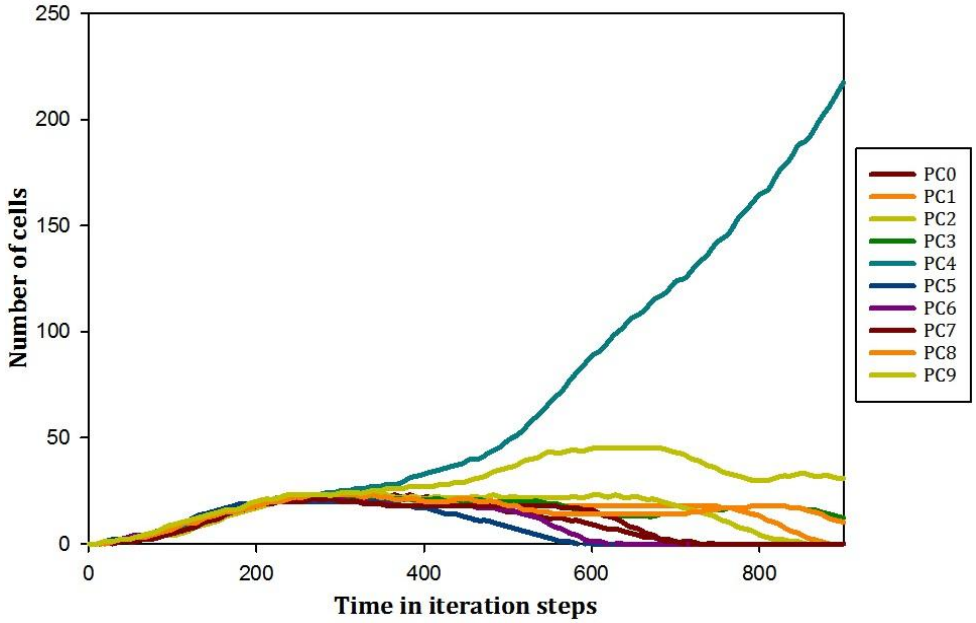


Figure 6.2 Number of cells from the 10 stem cell lineages as a function of time. Stem cell PC4 and its progeny (blue line) has an APC mutation that only affects the stem cell division function.

Perturbations to migration and stem cell division processes lead to steady increase of total cell number. Perturbations to stem cell division alone, increases only the number of cells in the stem cell niche. Perturbations to the other processes, though they increase the proliferating pool, do not result in increased total cell number since migration is normal and so cell clearance works normally. Perturbation of the cell shedding process alone does not seem to create significant accumulation of cells at the top. Mutated cells remain slightly longer at the top, but ultimately shed and leave the system.

6.3.2 Different combinations of perturbations

6.3.2.1 Perturbing migration and contact inhibition together

Table 6.3 shows that perturbation to migration reduces the proliferating pool of cells and pushes down the proliferating cell boundary. This could be because the contact inhibition of proliferation has been left unperturbed in this experiment. As migration slows down, cells crowd together. The normal contact inhibition that increases in a

crowded location would tend to keep the cells in the quiescent pool rather than in the proliferating pool.

Therefore we experimented with perturbations to both contact inhibition and migration processes together i.e. we used mutated APC_{conc} value in Eqn. (6.7) and Eqn. (6.8). We found that when contact inhibition was perturbed along with perturbing migration, the retention time reduced from 450 to around 350 iteration steps and the extent of proliferative zone became closer to the normal value of 25%.

6.3.2.2 Perturbing all processes except migration

The results show the total cell number steadily increasing as a function of time (Figure 6.3A). There is highly increased proliferation in the crypt, with the proliferating zone extending up to the top of the crypt. The steady increase in total cell number was unexpected because we anticipated that the migration process, being left normal, would clear the crypt and leave the cell number steady. We looked at the stem cell compartment to see if this unexpected increase of cell number is coming from the stem cell compartment. But we found that even though the mutated stem cell progeny had a clear advantage over other stem cell lineages (Figure 6.3B), the total stem cell numbers remained normal (Figure 6.3C).

We found the explanation for increasing number of cells when we looked at the distribution of cell numbers along the crypt axis (Figure 6.3D). We found that cells were accumulated near the top of the crypt. This showed that it was the perturbation to cell shedding that was increasing the cell numbers in the crypt. Perturbed stem cell division produces increasing number of mutated cells that migrate normally and reach the top. There they accumulate because the mutation slows down the shedding process. When we shut off the perturbation in cell shedding and allow the migrating cells to shed from the top, the total number of cells in the crypt remained steady. Thus though mutations that perturb the shedding process do not by themselves affect the homeostasis in the crypt (as seen in Table 6.3), they can lead to abnormal accumulation of cells in combination with mutations that perturb division and differentiation.

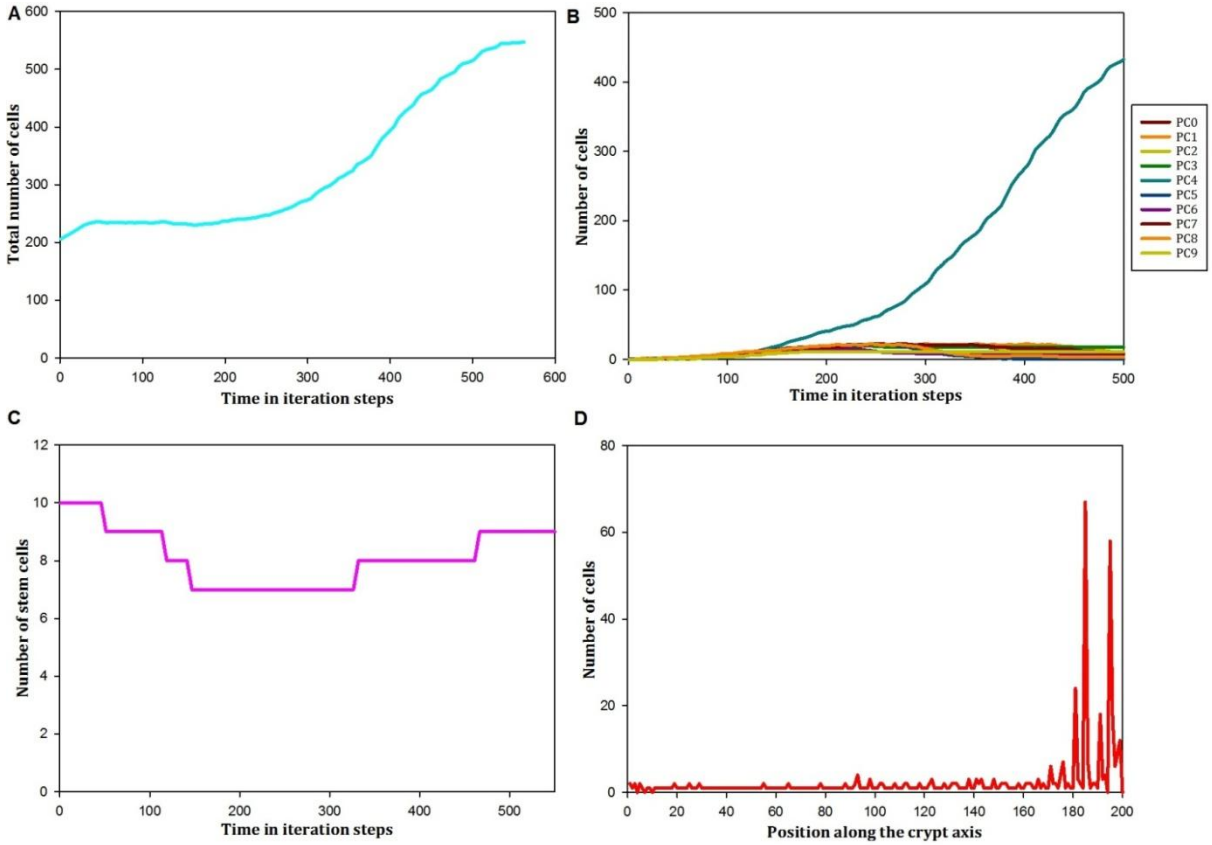


Figure 6.3 Consequences to the crypt when all processes except migration are perturbed. (A) The total number of cells in the crypt increases steadily as a function of time (B) Stem cell lineage PC4 (blue line) out grows all other lineages (C) Number of stem cells in the niche stays fairly constant (D) Distribution of cells along the crypt axis shows accumulation of cells at the top of the crypt

6.3.2.3 Perturbing all processes except migration and stem cell division

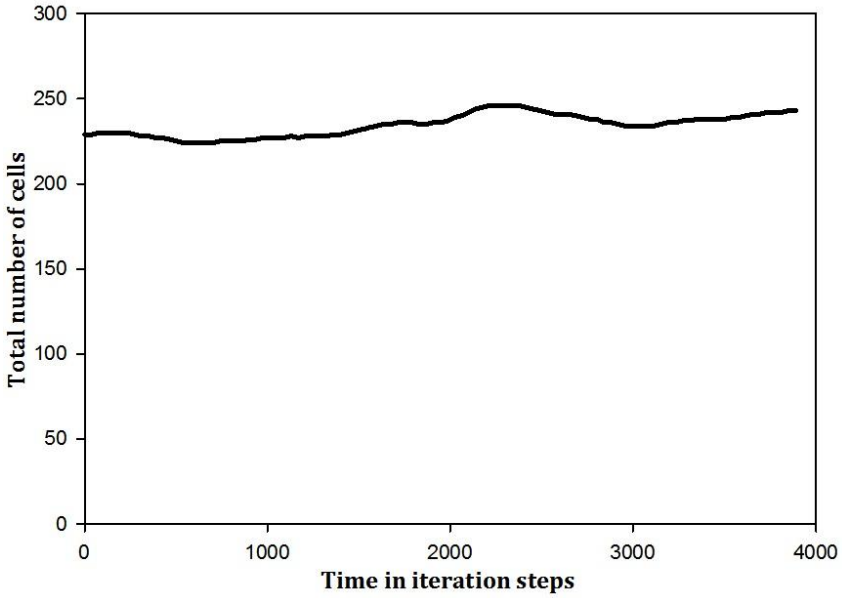


Figure 6.4 The total number of cells in the crypt as a function of time. One of the ancestral stem cells has a mutation that perturbs all the processes except migration and stem cell division

Figure 6.4 shows that the total cell number remains steady in this case. This is because the mutated stem cell lineage does not gain dominancy in the niche as the mutation does not bias the stem cell division in favor of the mutated stem cell. (Figure 6.5)

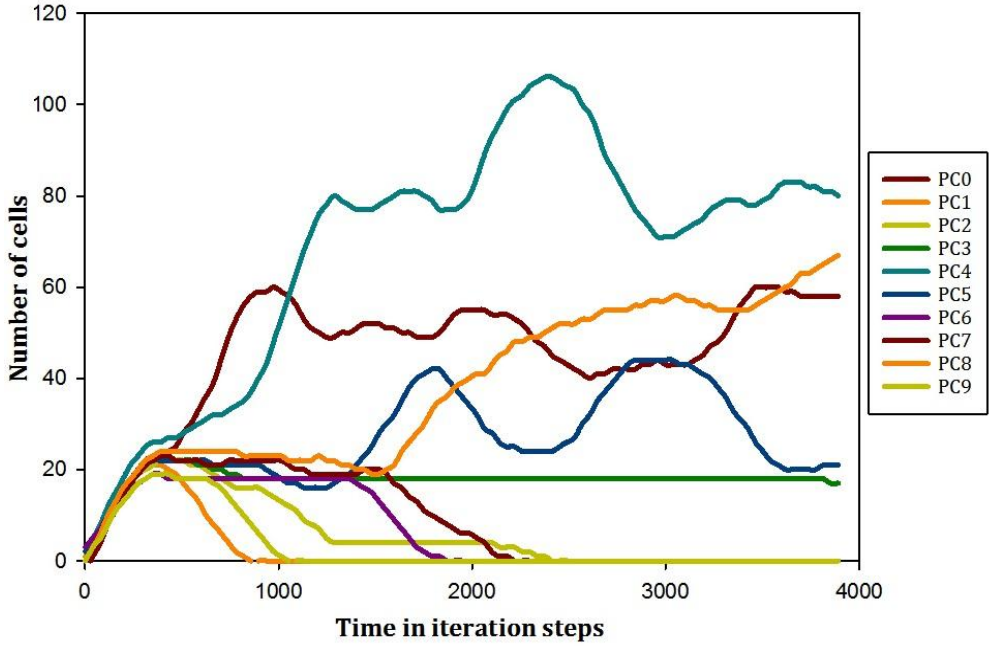


Figure 6.5 The 10 stem cell lineages as a function of time. Stem cell PC4 and its progeny (blue line) have an APC mutation that does not affect stem cell division or migration.

Without dominance in the niche, the mutated lineage does not get much dominance in the crypt also. Only a few mutated cells are slowed down at the top and so the cells do not accumulate at the top as it does in the case of 6.3.2.2

6.3.2.4 Perturbing all the processes together

On perturbing all the processes together (i.e. assuming that the mutation affects all the processes), we find that the mutated lineage comes to dominate the niche and the crypt. Migration is slowed down to such an extent that it could hardly be noticed in the simulations. The cells have high degree of stemness. The proliferative hierarchy in cell arrangement is lost and proliferative cells can be found throughout the crypt. The cell number increases steadily. The results are summarized in Table 6.4.

Table 6.4 All processes perturbed together

Indicators of change in the crypt	Unperturbed	All six processes perturbed
Retention time (iteration steps)	65 - 75	Mutated cells hardly move
Proliferative zone (% of crypt occupied by proliferating cells)	25%	Extends throughout
Proliferating cells (% of total number)	28%	72%
Survival Advantage to the mutated progeny	NA	Yes. Complete take over by mutated cells
Total Number of cells	224	Steady increase

6.4 Conclusions

The extended model of the crypt presented in this chapter has been shown to be capable of reproducing experimentally observed features of the APC mutated colon crypt, like larger pool of proliferating cells, loss of proliferative hierarchy in cell arrangement, more stem-like cells, and slowdown of migration. The tendency to form tumors on loss of APC is shown up in the simulation as a steady increase of cell number.

The model was used to perform several virtual experiments in order to understand the consequences of the different functional losses of APC. It was found that disturbances to migration and to the balance of stem cell division process have the highest impact on crypt homeostasis. None of the other perturbations by themselves, were found to result in steady increase of cell number (tumor formation). Slowing down of migration retains the mutated cell for longer periods in the tissue thereby enhancing its chances for acquiring additional mutations and progressing to full-fledged carcinoma. A mutated stem cell that has higher probability of self-renewal gets a selective advantage over the other stem cells and takes over the crypt by clonal conversion.

7

Conclusion

7.1 Summary

In this thesis we have presented the details of our investigation into the multiple roles played by Adenomatous Polyposis Coli in maintaining homeostasis in the colon crypt and the multiple ways in which functional losses of the APC protein can disturb the homeostasis and lead to initiation of colon cancer.

The main investigative tool used is computational modeling. Making use of available knowledge scattered in scientific literature we have developed rule based computational models and used these models as in-silico experimental facilities to generate more knowledge. Computational experiments have been performed for generating and testing new hypotheses or for asking what-if questions that lead to greater understanding of the phenomena investigated.

We developed models at two scales. Agent Based Modeling with cells as the agents, was used to model the dynamics of the colon crypt and Stochastic Simulation Modeling was used to model the Wnt/β-catenin signaling pathway that plays a major role in controlling the cellular processes at the molecular level. The APC molecule participated directly in the molecular scale model through its interactions with other molecules, whereas in the tissue scale model it participated indirectly through the rules of behaviour of the cell agents.

We first developed an Agent Based Model that could reproduce the dynamic processes of cell division, differentiation, migration and death, that maintain the homeostasis of the colon crypt. Observed features of the normal colon crypt like the random takeover of the crypt by a stem cell lineage (niche succession) were found to emerge naturally out of individual cell behaviors. Using this model we were able to propose and test a hypothesis on the manner in which APC mutations affect the stem cell division process.

We then investigated the multiple roles played by APC in the Wnt/β-catenin signaling pathway, which is a major molecular pathway that affects cell behavior in the colon epithelium. We studied the relative effects of five potential ways in which APC regulates Wnt/β-catenin signaling and concluded that APC's role in the destruction of cytoplasmic β-catenin has the highest impact on the regulation of Wnt/β-catenin signaling.

Finally an extended version of the Agent Based Model was developed that explicitly models the multiple roles played by APC in maintaining crypt fidelity and how perturbations to these roles due to APC mutations affect the overall features of the crypt. Observed features of APC mutated crypts were reproduced in simulations using the model. In-silico experiments queried the model with several what-if questions to separate out the consequences of each perturbation alone and in specified combinations. Perturbations to stem cell division and to cell migration were seen to be necessary conditions for carcinogenesis.

7.2 Future directions

The models developed in this work are essentially simple, using simple representations of the crypt geometry and simple, mostly linear, rules to describe the behavior of lower level components of the system. However they have been shown to reproduce complex features at the collective level and to serve as powerful platforms for in-silico experimental investigations. There are two directions in which this work can be expanded in future:

1. More in-silico experiments

   Only a few samples of the experimentation capability of the platforms have been demonstrated in this thesis. There is a huge scope for further in-silico investigations using these platforms. For example, crypt ABM presented in Chapter 6 can be used for studying the effects of different sequences of occurrence of the mutations, or the different consequences of a stem cell getting mutated, and a Transit Amplifying cell acquiring a mutation can be investigated. There are a large number of such possibilities.

2. Enhancement of the platforms

There is great scope for enhancement of the platforms themselves by way of improved rules, more accurate parameters and larger computational domains that more closely represents the real size and geometry of the crypt. As more and more experimental information becomes available, the rules and parameters of the models can be continuously improved. Use of more computational power and advanced programming techniques can enable better representation of actual crypts.

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LIST OF PUBLICATIONS

SCI Journals

1. Sasikumar R, **Rejitha JR**, Binumon PK, Manoj M (2011) Role of Heterozygous APC Mutation in Niche Succession and Initiation of Colorectal Cancer - A Computational Study. PLoS ONE 6(8): e22720. https://doi.org/10.1371/journal.pone.0022720

2. **Rejitha JR**, Roschen Sasikumar and Elizabeth Jacob (2018) Multiple Roles of Adenomatous Polyposis Coli gene in Wnt Signalling – a Computational Model.
   BioSystems (Revised Manuscript Submitted)

Conference Papers/Presentations/Posters

1. ***Best Poster Award*: Rejitha J.R** and Roschen Sasikumar. A Computational Investigation of the Role of APC gene in Colon Cancer Initiation. Poster Presented in the 2nd International Conference on Biotechnology & Bioinformatics (ICBB-2015) held at Pune from 6th to 8th February 2015.

2. **Rejitha J.R**, Binumon P.K, Roschen Sasikumar. Simulation of the dynamics of normal and abnormal colon crypt epithelium. Paper presented in 22nd Kerala Science Congress, held at Peechi, Thrissur from 28th to 31st January 2010.

3. **Rejitha J.R** and Roschen Sasikumar. Stochastic Simulation of the Multiple roles of APC in the Canonical Wnt Pathway. Poster Presentation at NextGen Genomics, Biology, Bioinformatics and Technologies (NGBT) Conference held at Cochin from October 3rd to 5th 2016.